BRAVO, A NOVEL IMMUNOGLOBULIN SUPERFAMILY MEMBER IN THE DEVELOPING AVIAN NERVOUS SYSTEM, IS IDENTIFIED USING A NEW METHOD

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Abstract

Cell-surface molecules play an essential role in guiding axons to their targets. We have developed a method to generate monoclonal antibodies (MAbs) which recognize cell-surface molecules of defined molecular weight that are expressed during the development of the chicken retinotectal system. The antigen distribution on optic fibers recognized by one of these MAbs, Bravo, is restricted to retinal ganglion cell axons in the retina, and absent from these same axons in the tectum.

The complete derived sequence of Bravo including four putative alternatively spliced regions has been obtained. It reveals a close relationship to the neural members of the immunoglobulin superfamily, with closest relationship to chicken Ng-CAM and mouse L1. Like Ng-CAM and L1, Bravo contains six immunoglobulin domains, five fibronectin type III repeats, a transmembrane domain and a highly conserved but functionally uncharacterized cytoplasmic region. Like the other neural members of the immunoglobulin superfamily, Bravo carries the HNK-1 carbohydrate epitope, and specifically like L1 and Ng-CAM, Bravo is found predominantly in the form of a heterodimer, an intact chain cleaved in identical locations in all three molecules into two non-covalently associating parts.

These data present an interesting view of retinotectal optic fiber outgrowth: As optic fibers grow in dense fascicles towards the optic nerve exit, they express both the known cell adhesion molecule Ng-CAM, and the closely related Bravo. As these fibers pass through the optic chiasm, Bravo is reduced on their surfaces, coincidentally in the same place and time that these fibers noticeably defasciculate, presumably to allow independent target seeking by individual axons.

Furthermore, Bravo staining of retinal glial processes has been detected. The close relationhip of Bravo to Ng-CAM coupled to Ng-CAM's known homophilic binding capacity suggest Bravo may be the heterophilic glial ligand for Ng-CAM long postulated to exist. Bravo is identical in sequence with the recently characterized Ng-CAM related glycoprotein, Nr-CAM.

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Chapter I--The Retinotectal System

Introduction

The development of a functioning nervous system involves the creation of a complex network of cells and cell processes. In vertebrates, this often requires neurons born in one area to project axons and form synapses in a distant area of the nervous system. The visual system of lower vertebrates is particularly well-suited to studies of patterned neuronal projections. In general, fibers from retinal ganglion cells grow towards the optic fissure and exit the retina as the optic nerve, which crosses the midline at the chiasm and innervates the optic tectum. A topographic map of the retina is formed on the tectum with neighboring retinal cells projecting to neighboring tectal cells. Anterior (nasal) retina projects to posterior (caudal) tectum, and posterior (temporal) retina projects to anterior (rostral) tectum. Dorsal retina projects to ventral tectum; ventral retina, to dorsal tectum. Thus, spatial relationships are maintained (though rotated) in the tectum.

Explanations and models for this specificity have occupied researchers for over a century. Even before the high degree of precision in the map was determined by histological and electrophysiological experiments, Santiago Ramon y Cajal postulated that chemical cues might be involved in axons finding their target tissues (Ramon y Cajal, 1960 translation of 1929 paper). This view was not immediately accepted, and much of the work that followed Ramon y Cajal's emphasized mechanical forces and guiding channels in directing nerve growth (reviewed in Hamburger, 1980). Against this decidedly non-molecular background, Roger Sperry's (1963) chemoaffinity theory is

especially noteworthy. This theory was based, in part, on his previous work (1943) that indicated that regenerating axons from the eyes of lower vertebrates grow back to their original sites in the tectum. More strikingly, by crushing and disrupting the optic nerve or by removing part of the retina in goldfish, Sperry (1963) and Attardi and Sperry (1963) showed that retinal fibers regrowing along identical "channels" passed by thousands of unoccupied tectal sites before synapsing in the appropriate regions. More recent experiments support and expand upon this early work (Fraser and Hunt, 1980; Cowan and Hunt, 1985) including experiments which reveal the ability of axons to find their targets after transplantations (Yoon, 1971) and the re-establishment of proper connections following forced misrouting (Thanos et al., 1984). Taken together, these experiments strongly support the notion that fibers carry and can read intrinsic spatial cues.

Sperry proposed that "growing axons recognize their target cells by individual cytochemical identification tags," formalized in the chemoaffinity hypothesis (Sperry, 1963). Different researchers and modelers have interpreted the meaning of Sperry's theory in significantly different ways, though supporting statements by Sperry reveal with some clarity that he meant to propose an orthogonal gradient with matching "latitude and longitude" values expressed as a "chemical code" (quoted from Sperry, 1963). Regardless of the exact nature of Sperry's proposal, and it is not a refined model, the notion of chemical cues guiding nerve growth now forms the basis of all successful models of neural specificity, though several other mechanisms are clearly at work.

Neuronal activity also plays a role in the formation of the retinotectal projection, though this role appears to be limited to secondary refinements of the map. This notion is supported by

the regeneration of crude retinotectal maps in lower vertebrates deprived of normal neuronal activity by blocking with tetrodotoxin (Hartlieb and Stuermer, 1989; Schmidt and Edwards, 1983) or in animals raised under strobe lights so that all ganglion cells, not just close neighbors, fire at the same time (Schmidt and Eisele, 1985).

Furthermore, a great deal of plasticity is observed in this system. Amphibians and fish not only regenerate lost connections but also show continuous and asymmetric growth of the retina and tectum throughout life, forcing a gradual shift in neuronal connections in the tecta of these animals to maintain retinotopy throughout life (reviewed in Cline, 1991).

This plasticity has been pursued experimentally in goldfish (Schmidt et al., 1978; Yoon, 1971) and in *Xenopus* (Fraser and Hunt, 1978). After removal of part of the retina, the remaining retinal fibers grow to their appropriate targets at first, then slowly expand and fill up the empty parts of the tectum. Likewise, when whole retina are confronted with partial tecta, compressed maps are formed. Because these results appear so incongruous with the notion of chemically labeled optic fibers and target sites, some theoretical models have been proposed that almost completely abandon the role of intrinsic spatial cues in setting up the retinotectal projection. These models (*e.g.*, von der Marlsberg and Willshaw, 1977), however, fail to account for all the results noted above. Alternatively, Gierer (1983) has proposed that expansions and compressions can be explained on top of a "chemoaffinity" model if the density of retinal fibers can induce changes in the concentration gradient of the tectal cues. While such a model predicts normal innervation, as well as the results from tectal grafts and expansions and compressions of the map, the mechanism of retinal respecification of tectal markers seems problematic.

According to another model, both the specificity and the plasticity of this system can be explained without invoking respecification of the tectum (Fraser and Hunt, 1980). This model stipulates that chemoaffinity represents a much weaker force than the general repulsiveness of retinal axons to each other. Thus this model predicts the observed expansion of the partial retinal projection. A very strong general adhesiveness of retinal fibers for the tectum in this model ensures that it predicts compressions of the map as well. One additional advantage of this model is that it predicts the important role of homophilic adhesion molecules (many of which have been found) as well as molecules involved in specifying spatial position.

Models in general seek parsimonious solutions to biological problems, often revealing that a small number of simple interactions can explain an apparently perplexing phenomenon. One of the disadvantages of a reliance on models, however, is that they tend to promote the view that theoretically "clumsy" solutions (*i.e.*, requiring a large number of molecules) are consequently biologically unlikely (Gierer, 1983). Minimalist efficiency, however, is not always the rule in metazoan systems, especially in the developing nervous system where robust fidelity in the specification of connections is of such high priority.

This chapter will briefly review some of the proteins at work in facilitating the development of the retinotectal projection with an emphasis on more recent work, suggesting a greater diversity of these molecules than first suspected. The large diversity of molecules (some apparently redundant) so far identified within the developing retinotectal system in chickens highlights the distinction between computer versus biologically generated morphogenesis. That is, that the simplicity implied by some computer models is at best mediated by a complex set of biological molecules and interactions.

Adhesion Molecules

N-CAM and Related Molecules

Several classes of proteins that regulate the outgrowth of axons on cell surfaces and extracellular substrates have been identified, and the major ones will be briefly described here. Most of the molecules identified so far appear to mediate cell-cell and cell-substrate adhesion rather than repulsion, and many of these molecules are related to each other and to the neural cell adhesion molecule, N-CAM. N-CAM appears early in development and is expressed through to adulthood on neurons in chicken (Edelman, 1986). It promotes homophilic cell-cell adhesion via a Ca^{++}-independent mechanism and appears to be involved in axon-axon adhesion, neuroblast migration, stabilization of synaptic junctions and varied roles in other tissues (Edelman, 1986). N-CAM appears in muscle, kidney, heart and in a strikingly ordered fashion in developing chicken feathers (Crossin et al., 1985; Chuong and Edelman, 1985).

N-CAM is expressed in a wide variety of isoforms generated by alternative mRNA splicing of a single 50 kb gene (Murray et al., 1986). Each N-CAM isoform has five domains homologous to immunoglobulin-like domains of category C2 (Williams and Barclay, 1988) followed by two regions resembling fibronectin type III repeats. Major differences in N-CAM forms relate to the mode of membrane attachment and to the cytoplasmic domains. N-CAM 180 (also called N-CAM ld for "large cytoplasmic domain") and N-CAM 140 (also called N-CAM sd for "short cytoplasmic domain") are both integral membrane proteins with identical extracellular domains and a cytoplasmic region either containing or missing a 261 amino acid ld exon. N-CAM 120 (also called ssd for "small surface domain") is the smallest form of N-CAM and is

attached to the membrane by a phosphatidylinositol linkage. This Id sequence has been shown to interact with brain spectrin, a component of the cytoskeleton (Pollerberg et al.,1987).

The possible role of cytoskeletal attachment in N-CAM mediated adhesion has been further supported by studies in which cells were transfected with N-CAM and/or chimeric molecules consisting of a homophilic extracellular domain coupled to the N-CAM Id cytoplasmic domain. Cells transfected with these molecules showed extensive aggregation in controls and when treated with cytochalasin D (which disrupts actin filaments), but showed significantly reduced aggregation when treated with nocodazole (which inhibits microtubule assembly) (Jaffe et al., 1990).

In addition to the mode of membrane anchoring, diversity of N-CAM forms is created in a variety of ways. A growing number of different putative alternatively spliced forms of N-CAM are being identified by cDNA and genomic analyses, and many of these alternative exons occur in the extracellular portion of the molecule and Ig-like domains themselves, suggesting a role for alternative splicing in modulating N-CAM adhesion (Santoni et al., 1989; Small et al., 1988; Reyes et al., 1991). A muscle-specific form of N-CAM has been found to encode a 31 amino acid long exon not found in neural tissues, but similar in sequence to the ssd region (Prediger et al., 1988). The spatio-temporal distribution and possible role of these different isoforms has not yet been established.

Besides alternative splicing, other mechanisms increase the diversity of N-CAM form and function. N-CAM has been shown to interact with heparan sulfate proteoglycan which may affect homophilic or substrate binding (Cole and Glaser, 1986). Also, Adult (A) and Embryonic (E) forms of N-CAM differ significantly in their extent of glycosylation. The fifth Ig-like domain

contains several sites for the attachment of an unusual and highly anionic oligosacharide (alpha-2,8-polysialic acid) (PSA) which appears to inhibit cell binding when present at high levels in the E form (Hoffman et al., 1982; Crossin et al., 1984). A shift from E to A forms seems to coincide with cessation of cell migration and axon outgrowth in the mouse cerebellar cortex (Hekmat et al., 1990). Analogously, in the chicken retinotectal system, distal and growing tips of retinal ganglion axons express the E form, while on more proximal parts the less glycosylated (and presumably more adhesive) A form is found (Schlosshauer et al., 1984).

A number of other glycoproteins are found in the developing nervous system which contain multiple C2 category Ig domains and fibronectin type III homology units (reviewed in Jessell, 1988). These are: Fasciclin II in *drosophila* (Harrelson and Goodman, 1988); L1 in mouse (Moos et al., 1988) in rat (Prince et al.,1989; Miura et al., 1991) and in human (Djabali et al., 1990) and a related molecule, neuroglian, in *drosophila* (Bieber et al., 1989); Ng-CAM in chicken (Burgoon et al., 1991); Contactin/F11 in chicken (Ranscht, 1988; Brummendorf et al., 1989) and called F3 in mouse (Gennarini et al., 1989); and TAG-1 in rat (Furley et al., 1990) and preliminarily identified as axonin-1 in chicken (Ruegg et al., 1989, and P. Sonderegger, personal communication).

While none of these molecules have been analyzed with any degree of quality at the level of crystal structure, they show meaningful sequence similarity with immunoglobulin domains (especially at sites known to confer proper folding of these domains) and are thus included in the immunoglobulin superfamily (Williams and Barclay, 1988). Computer analyses and alignments of these with other immunoglobulin superfamily members suggest that the Ig domains in N-CAM and related molecules contain seven β sheets resembling immunoglobulin constant (C) domains, while

paradoxically, the sequences themselves more closely resemble immunoglobulin variable (V) regions. Hunkapiller and Hood (1989) have suggested that the distinct nature of these neural Ig domains justifies a separate category "H," but as this name is not widely used, I refer to them by William and Barclay's (1988) term, C2.

These N-CAM related molecules are found on post-mitotic cells in the developing nervous system but not on cells in early embryos. These molecules are prevalent on axons, especially of the developing retinotectal system in chickens, and Rutishauser has used the term Ax-CAMs to highlight this fact (U. Rutishauser, personal communication). Antibodies against these molecules and against N-CAM generally disrupt fasciculation (Ruegg et al., 1989; Rathjen et al., 1987a; Fischer et al., 1986; and Edelman, 1986). Ng-CAM coated covaspheres aggregate, and this aggregation is inhibitable by anti-Ng-CAM antibody fragments revealing a role for Ng-CAM, like N-CAM, in homophilic cell adhesion (Grumet et al., 1984a). In addition, Ng-CAM has been shown to bind an unidentified molecule on glia (Grumet et al., 1984b; Grumet and Edelman, 1988). Finally, purified Ng-CAM (and not N-CAM) acts as a potent substrate for neurite outgrowth (Lagenaur and Lemmon, 1987).

Antibodies against another axonal glycoprotein, neurofascin, likewise disrupt fasciculation in retinal ganglion axons, so the possibility that this molecule is related to the N-CAM family is being actively pursued (Rathjen et al., 1987b, and personal communication). Though the functions and binding properties of most of these Ax-CAMs (discussed further in Chapter IV) are poorly understood, their patterns of distribution in the developing retinotectal system and their structures and relationships to other molecules provide possible clues to their roles.

Cadherins

While N-CAM and N-CAM related molecules mediate cell binding in a Ca^{++}-independent manner, it has long been recognized that Ca^{++} is essential in most systems for maintaining cell-cell contacts. Molecules involved in this Ca^{++}-dependent adhesion are called Ca^{++}-dependent CAMs by Edelman and others, but the lack of any homology to the Ig-related CAMs prompts the use here of the preferred term, cadherins. Members of the cadherin family show generally higher levels of amino acid conservation, around 50%, than do members of the Ig-related CAM family (reviewed in Takeichi, 1990).

The N-terminal portion of the cadherins contains four extracellular repeats with short conserved sequences. The most N-terminal region exhibits the conserved sequence His-Ala-Val (HAV) common to all the classical cadherins: E-(also called uvomorulin and homologous to the chicken L-CAM), P-, N-(also called A-CAM), R- and B-cadherins (Napolitano et al., 1991; Inuzuka et al., 1991; Takeichi, 1991). Embryo compaction and neurite outgrowth are found to be developmental functions blocked by anti-cadherin antibodies *in vivo* and inhibitable by HAV containing peptides (Blaschuk et al., 1990). Mouse L-cells transfected with different cadherins form separate and homogeneous aggregates in cell aggregation studies (Takeichi et al., 1985). In addition, cell transfection experiments reveal the importance of extreme N-terminal regions in cadherin mediated cell binding. Moreover, site specific mutagenesis reveals that the two amino acid positions surrounding the HAV sequence (variable residues at the underlined positions, $\underline{X}$HAV$X\underline{X}$) are involved in the specificity of the adhesive response (Nose et al., 1990).

These transfection and aggregation experiments reveal a homophilic mode of action for cadherins localized to the N-

terminus of the molecule. Therefore it is not surprising that most MAbs that inhibit Ca^{++}-dependent cell-cell adhesion bind to cadherins at the N-terminus. More recently, an antibody that blocks E-cadherin mediated adhesion was found to bind the extracellular anchor portion of the molecule, immediately N-terminal to the transmembrane domain (Ozawa et al., 1990a). This region contains four cysteines at conserved locations in the different cadherins, and a role for disulphide bonding in affecting adhesion is suggested by the fact that reduction of these bonds by dithiothreitol reduces the adhesiveness of cells expressing E-cadherin.

Cell adhesion is dependent upon not only the extracellular binding and cysteine rich regions of these molecules, but also, and profoundly, by their cytoplasmic domain. Perhaps this is not surprising given the 90% conservation of amino acid sequence in this region. Unlike N-CAM and Ng-CAM for example, which can affect homophilic aggregation of synthetic beads or liposomes, cadherins can not, suggesting a requisite interaction with the cytoplasm, and probably with the cyto-architecture, to function properly. To analyze this interaction, deletion experiments with E-cadherin constructs transfected into mouse L-cells were performed. These experiments reveal that the most C-terminal sequences are required for cadherin mediated adhesion. Furthermore, these sequences are necessary for cadherin binding to a group of ubiquitous cytoplasmic proteins called catenins (Ozawa et al., 1990b). The complex nature of catenin association with actin based cytoskeleton, and in general of the mechanism of cadherin-cytoskeleton interactions and the role of cadherins in transducing extracellular information to the cell, are poorly understood but actively pursued at this time.

More relevant to this discussion is the possible role cadherins play in development. The homophilic mode of action of

these molecules combined with their pattern of distribution suggest a role in maintaining ordered associations among like cells. This role is strongly implied by the spatio-temporal pattern of cadherin expression in the developing chicken embryo (reviewed in Takeichi, 1990). In the formation of the neural tube, E-cadherin is turned off in the neural plate and replaced by N-cadherin simultaneous with the separation of the plate from overlying (E-cadherin positive) ectoderm. This correlation between sorting of cell layers and expression of different cadherins is seen in other regions in the chicken, and in mammalian developmental processes as well.

The relatively simple view that cadherins are involved in tissue-specific cell sorting events is currently being modified to account for more recent results. For example, a retinal cadherin, R-cadherin, has been shown to have a weak heterophilic affinity for the closely related N-cadherin, in addition to the expected high homophilic affinity (Inuzuka et al., 1991). Also, a number of cadherin related cDNAs have been generated using the polymerase chain reaction and conserved primer sequences, and most of these are expressed in the nervous system (Suzuki et al., 1991). The distribution of these new and putative cadherins will suggest whether or not cadherins play a role not only in cell-type and tissue organization, but also possibly in regional spatial affinities of cells for their neighbors (discussed in Inuzaka et al., 1991).

Integrins and Extracellular Matrix Molecules

A third class of molecules known functionally as substratum adhesion molecules (SAMs) mediates interaction between neurons and the extracellular matrix (ECM). Most of these molecules have been discovered to be a family of cell-

surface receptors now referred to as integrins (reviewed in Reichardt, 1991). Integrins are, in general, heterodimers, an α subunit thought to bind Ca^{++}, noncovalently associated with a β subunit with four characteristic cysteine-rich repeats. Each chain has a transmembrane domain and short cytoplasmic sequences that are involved in the indirect linkage of integrins with the cytoskeleton. At least eleven α chains are known to exist and associate with at least six β chains (Hynes, 1990; Albeda and Buck, 1990). At first, it was thought that each α chain associated with only one β chain, but new evidence has shown that this is not always the case. For example, α_v combines not only with β_2 to form the classic vitronectin receptor, but also with β_1 (Vogel et al., 1990) and β_6 (Cheresh et al., 1989). It therefore seems possible that a tremendous variety of integrins could exist, created by the shuffling of α and β chains.

The substrates for this diverse family of receptors are equally diverse, and cannot be completely reviewed here. In general, these ECM components are composed of regions with extensive internal homology repeats and homologies with other known proteins. For example, the amino acid sequence of the ECM component, tenascin (also called cytotactin), reveals a region with homology to fibrinogen, several fibronectin type III repeats, a series of epidermal growth factor-like repeats , several heptad repeats associated with alpha helices of ECM proteins, and one unique terminal region involved in tenascin's multimeric assembly.

Often these modular or "mosaic" (Doolittle, 1985) proteins are combined to form diverse multimers. Laminin, for example, has five subunits combined in four known ways to produce distinct laminin tetramers (Sanes et al., 1990). Laminin is found in regions of nerve growth and, in culture, is a powerful promoter of neurite outgrowth (Liesi et al., 1984) .

Fibronectin, by far the best studied of the ECM components, is a dimer, each component consisting of an assembly of three different types (I, II, and III) of repeating modules. Fibronectin type I module is involved in heparin binding and matrix assembly; type II, in collagen and gelatin binding; and type III repeats, found in many other ECM molecules as well as CAMs, muscle proteins and even the laminin receptor integrin, is involved most significantly in cell binding (Hynes, 1990; Suzuki and Naitoh, 1990; Hogervost et al., 1990). Type III repeats as functional domains will be discussed more extensively in Chapter IV with respect to their presence in the Ig-related glycoprotein, Bravo. Alternative splicing, rather than subunit shuffling as with laminin, generates fibronectin diversity.

Thrombospondin and vitronectin are ECM components of the developing retina, and promote neurite outgrowth *in vitro* (Neugebauer et al., 1991). Many more ECM components have been analyzed including several different types of collagen, and most, when they affect nerve growth at all, do so by promoting cell attachment and neurite outgrowth (or cell migration).

The mechanism of this growth promoting activity and the role of integrins is beginning to be understood. For years, evidence has been accumulating that integrins are associated (and co-purify) with not only ECM molecules but also cytoskeletal components such as actin, talin and vinculin (Chen et al., 1985; Horvitz et al., 1986; Otey et al., 1990). A role for cytoskeletal-integrin linkage and a resulting physical cell tension mediating directed nerve growth has been postulated (Mitchinson and Kirschner, 1988; Letourneau and Shattuck, 1989; Heidmann and Buxbaur, 1990). Also neurite outgrowth on laminin, type IV collagen and fibronectin are reduced in the presence of protein kinase C inhibitors (though outgrowth on purified Ng-CAM is not),

suggesting an active role for PKC phosphorylation in integrin function (Bixby and Jhabvala, 1990).

Not all ECM components promote cell adhesion, some "anti-adhesive" proteins have been described. The SPARC glycoprotein is a common component of basement membranes that when added to cell cultures inhibits cell spreading (Sage et al., 1989). Tenascin, mentioned above among the neurite-promoting ECM molecules, has a separate anti-spreading effect on neurons (Faissner and Kruse, 1990; Spring and Chiquet-Ehrismann, 1989). The mode of action of the molecules and for others that mediate cell-substratum and cell-cell repulsion (reviewed in Patterson, 1988; Keynes and Cook, 1990), are not yet known; however, some data on one such molecule has been forthcoming and is discussed below.

Specificity Molecules?

Graded Molecules

The molecules discussed above certainly play a vital role in neural morphogenesis: in mediating neuronal and glial differentiation, proliferation, migration, adhesion and cell death; in affecting axonal outgrowth and guidance away from certain regions and towards others; and in growth cone recognition and synapse formation at certain target cell types and tissues. No evidence yet exists, however, to suggest that these molecules contribute to specifying retinotectal position. The extensive spatial distribution of these molecules is not consistent with the specification of place. At a molecular level, however, a great deal of heterogeneity (*e.g.*, a spatially restricted distribution of alternatively spliced isoforms or post-translational

modifications) may exist that the methods (*e.g.,* antibodies directed against common protein epitopes) fail to detect.

Some molecules known to be spatially distributed in the retinotectal system are known to exist. One way position can be specified, as discussed above, is through the use of molecular gradients. The JONES antibody labels the murine retina in a dorsoventral gradient during development (Consantine-Paton et al., 1986). The JONES antigen is a carbohydrate epitope of the GD3 ganglioside and may be identical to D1.1 (Levine et al., 1984). Though increasing evidence suggests that carbohydrates, especially gangliosides, are involved in cell adhesion of neurons (Jessell et al., 1990), the difficulty of analyzing and purifying these molecules has hindered discovery in this field.

More tractable has been the discovery and analyses of two protein molecules with graded distributions identified in the retina and tectum by Trisler et al. (1981). TOP_{DV} is a 47 kD cell-surface protein distributed on all cells of the retina in a dorsoventral gradient, with higher levels in the dorsal retina. During development, a 3-fold distribution of dorsal versus ventral TOP_{DV} at E4 increases to an 8-fold difference by E8 (when most retinal axons are seeking targets in the tectum) growing to a 35-fold gradient at hatching. TOP_{DV} is also found in the tectum, even before retinal fibers arrive, in a gradient inverted with respect to the retinal gradient. The level of TOP_{DV} on a particular cell or fiber is therefore a marker of its dorsoventral position, both in the retina and tectum (Trisler et al., 1981).

A similar case can be made for TOP_{AP}, a 40 kD cell-surface molecule distributed in a gradient of lower magnitude than TOP_{DV} in both the retina and tectum (Trisler, 1990). TOP_{AP} is found at 16-fold higher levels in the peripheral posterior (temporal) retina than in the corresponding anterior (nasal) retina. Likewise, the polarity of this gradient is reversed in the tectum.

The TOP molecules present an orthogonally graded coordinate system that might be used to identify cell position in the retina and tectum (Sperry, 1963). Furthermore, the inversion of the orientation of the gradients in the tectum corresponds to the rotation of the retinal map. Axons with high levels of TOP_{DV} (dorsal retina) synapse on tectal cells with correspondingly high levels of this same molecule (ventral tectum), and vice versa. Similarly, axons with high levels of TOP_{AP} (posterior retina) project to tectal cells with high levels of the same protein (anterior tectum), and vice versa. Thus the distribution of TOP molecules conforms to those predicted for graded homophilic positional markers (Fraser and Hunt, 1980).

The gene for TOP_{AP} has been partially cloned, and sequence data is forthcoming; however, no evidence yet exists to suggest these molecules function in homophilic adhesion. Antibody perturbation experiments *in ovo* show that synapse formation is delayed in the retina in the presence of anti-TOP_{DV}. While this inhibition is consistent with a view of TOP_{DV} as being involved in recognition that must precede synapse formation, it is far from conclusive. The graded distribution of TOP molecules is suggestive of a role in retinotectal specificity that the characterization of these proteins and their functions is hotly anticipated. However, other molecules unlikely to be involved in positional specification, such as the murine rod opsin gene (Lem et al., 1991), have been detected in spatial gradients as well.

Another molecule, the function of which is better characterized, has been identified in the developing optic tectum by Bonhoeffer and his colleagues using an *in vitro* assay in which retinal axons are presented with stripes of different substrates on which to grow. The preferential growth of temporal retinal axons on stripes of more anterior tectal membranes had been previously shown to result from a graded repulsive molecule

rather than an attractive one because heat or enzyme treatment of the repellent posterior tectal membrane stripes abolished this preference (Bonhoeffer and Huf, 1980; Walter et al., 1987a). In control experiments, these treatments of anterior tectal membranes had no effect.

Using a novel assay developed by Raper and Kapfhammer (1990), Cox et al. (1990) recently showed that the induction of growth cone collapse is likely to be the mode of action of this repulsive guidance. Müller et al. (1990) has now identified a 33 kD, glycosyl-phosphatidylinositol linked glycoprotein distributed in an anteroposterior gradient suggestive of its role in affecting growth cone collapse and resulting axon guidance. Vesicles containing the 33 kD glycoprotein behave similarly to native posterior tectal membranes in both the stripe assay and the growth cone collapse assay, and the activity of these vesicles is destroyed by heat; by treatment with phospholipase C; by treatment with peanut lectin; and by treatment with antisera against posterior tectum. All of these treatments destroy the guidance and growth cone collapsing activities of native tectal membranes as well (Davies et al., 1990; Müller et al., 1990; Walter et al., 1987a and 1987b; Vielmetter et al., 1991). The determination of whether or not this 33 kD molecule affects growth cone collapse is likely to be soon achieved as MAbs against this 33 kD molecule have recently been generated (U. Schwarz, personal communication).

The role of this molecule in causing growth cone collapse *in vitro* seems probable, and its graded distribution suggests a role in retinotectal specificity. Unfortunately, the data accumulated thus far does not fit well with a simple gradient model. Nasal fibers, for example, do not respond at all to this molecule or to any of the growth inhibiting properties of the posterior tectum, and although temporal fibers avoid more posterior membranes in

stripe assays, when no choice is given, both temporal and nasal fibers grow on posterior membranes at the same rate (Walter et al., 1987b). Moreover, temporal fibers do not show a graded sensitivity to this molecule. In other words, the 33 kD molecule is indeed graded in the tectum, and this gradient is associated with a greater growth cone collapsing and repulsive activity in more posterior tectal membranes, but the temporal retinal fibers themselves do not show any heterogeneity in their response to this gradient. Central and peripheral temporal axons behave identically.

To account for these results, it has been postulated that the 33 kD molecule functions in "steering" all temporal fibers away from posterior tectal membranes. Alternatively, the *in vitro* assays may fail to reveal the effects of this molecule in the context of a complex cell-surface with multiple adhesive and repulsive interactions. The failure of temporal axons to sort out on graded distributions of posterior membranes (or vesicles containing the 33 kD molecule) might reflect the absence *in vitro* of normal competitive or repulsive interactions between retinal fibers. A graded distribution of sensitivity to the 33 kD molecule may exist in the temporal retina but not be detected in the stripe assays. Under this model, the lack of normal competition for substrate and soluble factors in culture combined with the fact that all temporal fibers have some sensitivity for the repulsive molecule, results in all temporal axons (regardless of the magnitude of their sensitivity) avoiding regions of high repulsive activity. *In vivo*, if more peripheral temporal axons have a higher sensitivity for the 33 kD molecule than more central temporal axons, then the graded distribution of the 33 kD molecule in the tectum in combination with a general axon-axon repulsion and competition for sites would generate an anteroposterior map, at least for temporal axons. Of tremendous benefit towards

addressing these questions will be the identification of the receptor for the 33 kD molecule, and the analysis of its graded or even distribution on temporal axons.

Compartmentalized Molecules

Besides graded molecules, another type of distribution capable of affecting positional specificity is a combinatorial or area-code molecule (Hood et al., 1977). Several molecules discussed above such as N-CAM, L1 and R-Cadherin can be viewed as combinatorially specifying cell and tissue type. More to the point of retinotectal specificity, a number of molecules asymmetrically distributed in the retina have been reported, including the cytoplasmic protein p40 originally thought to be a high affinity laminin receptor (McCaffery et al., 1990; Rabacchi et al., 1990) and an aldehyde dehydrogenase similar to those involved in retinoic acid production (McCaffery et al., 1991). In addition, TRAP (temporal retinal axon protein) distinguishes temporal retinal fibers from nasal ones in an on/off manner (McLoon, 1991). Temporal axons display the antigen, nasal ones do not. (The small number that do are postulated to be displaced ganglion cells described by Karten et al. (1977). Also, the 10/22A8 antigen that we've identified (Appendix II) is found abundantly on nasal retinal axons and nearly absent on temporal axons at E8. Unlike TRAP, this molecule is also found in both the inner synaptic layer and outer synaptic layer.

A distinction has been made here between molecules expressed with compartmental boundaries like TRAP and 10/22A8 and molecules expressed in a graded fashion like TOP_{DV}. The difficulty of quantitating protein levels immunohistologically coupled with the various word choices of different reports (*e.g.*,

"step gradient") obscures this distinction in the literature, but it is an important one for inferring function.

Unfortunately, little is known of the structure and possible function of these molecules. TRAP has an apparent molecular weight of 135 kD placing it in the size range, though not necessarily functional category, of many N-CAM related molecules (see Chapter IV). 10/22A8, also in this size range, has been partially sequenced and contains both immunoglobulin domains and fibronectin type III repeats, found on a variety of proteins including cell adhesion molecules. If these molecules are homophilically adhesive in nature, a simple prediction would have them involved in the selective fasciculation of temporal versus nasal axons, helping to ensure that all fibers exit at the fissure during development. Alternatively, McLoon has suggested that a combinatorial code of molecules like those combined with TOP_{DV} and a third gradient molecule (a hypothetical central to peripheral marker) could uniquely specify cell position. Although TOP_{AP} more readily combines with TOP_{DV} to specify position, the two coordinate systems are not mutually exclusive. Furthermore, the notion of a graded central-peripheral substance is indirectly suggested by the observation that some amacrine cells within the ventral retina send processes horizontally to mirror symmetrical regions of the dorsal retina. That is, central ventral amacrine cells send processes to central dorsal locations, and peripheral ventral amacrine cells to peripheral dorsal locations (Catsicas et al., 1987), possibly relying on a central to peripheral molecular gradient.

Other possible functions for such compartmentalized molecules include the proposal by Bonhoeffer and Gierer (1984) that a "step gradient" of a primary substance (*e.g.*, aldehyde dehydrogenase) can give rise to a smooth gradient of a secondary substance (*e.g.*, TOP_{DV}). Also, the distribution of TRAP is

consistent with the proposed distribution of a receptor for the 33 kD growth cone collapsing molecule described above. If these two molecules interact it would highlight both the distinction between graded and compartmentalized molecules and the fact that systems using each type of molecule can overlap.

References

Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB J.* 4:2868-2880.

Attardi, D. G., and R. W. Sperry. 1963. Preferential selection of central pathways by regenerating optic fibers. *Exptl. Neurol.* 7:46-64.

Bieber, A. J., P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. 1989. *Drosophila* neuroglian: A member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell* 59: 447-460.

Bixby, J. L., and P. Jhabvala. 1990. Extracellular matrix molecules and cell adhesion molecules induce neurites through different mechanisms. *J. Cell Biol.* 111: 2725-2732.

Blaschuk, O. W., R. Sullivan, S. David, and Y. Pouliot. 1990. Identification of a Cadherin cell adhesion recognition sequence. *Dev. Biol.* 139:227-229.

Bonhoeffer, F., and A. Gierer. 1984. How do retinal axons find their targets on the tectum? *Trends Neurosci.* 7: 378-381.

Bonhoeffer, F., and J. Huf. 1980. Recognition of cell types by axonal growth cones in vitro. *Nature* 288:162-164.

Brummendorf, T., J. M. Wolff, R. Frank, and F. G. Rathjen. 1989. Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. *Neuron* 2:1351-1361.

Burgoon, M. P., M. Grumet, V. Mauro, G. M. Edelman, and B. A. Cunningham. 1991 Mar. Structure of the chicken neuron-glia cell adhesion molecule, Ng-CAM: Origin of the polypeptides and relation to the Ig superfamily. *J Cell Biol.* 112:1017-29.

Catsicas, S., M. Catsicas, and P. G. H. Clarke. 1987. Long-distance intraretinal connections in birds. *Nature* 326:186-187.

Chen, W. -T, E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in culture fibroblasts. *J. Cell Biol.* 100: 1103-1114.

Cheresh, D. A., J. W. Smith, H. M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin ($\alpha v \beta x$) is responsible for distinct adhesive properties of carcinoma cells. *Cell* 57:59-69.

Chuong, C. M., and G. M. Edelman. 1985. Expression of cell-adhesion molecules in embryonic induction II: Morphogenesis of adult feathers. *J Cell Biol.* 101: 1027-43.

Cline, H. T. 1991. Activity-dependent plasticity in the visual systems of frogs and fish. *Trends Neurosci.* 14:104-111.

Cole, G. J., and L. Glaser. 1986. A heparin-binding domain from N-CAM is involved in neural cell-substratum adhesion. *J. Cell Biol.* 102:103-112.

Cole, G. J., and M. Schachner. 1987. Localization of the L2 monoclonal antibody binding site on chicken neural cell adhesion molecule (NCAM) and evidence for its role in NCAM-mediated cell adhesion. *Neurosci. Lett.* 78: 227-232.

Constantine-Paton, M., A. S. Blum, R. Mendez-Otero, and C. J. Barnstable. 1986. A cell surface molecule distributed in a dorsoventral gradient in the perinatal rat retina. *Nature* 324:459-461.

Cowan, W. M., and R. K. Hunt. 1985. The Development of the Retinotectal Projection: An Overview. *In* Molecular Bases of Neural Development. G. M. Edelman, W. E. Gall, and W. M. Cowan, eds. John Wiley and Sons, New York.

389-428.

Cox, E. C., B. Müller, and F. Bonhoeffer. 1990. Axonal guidance in the chick visual system: Posterior tectal membranes induce collapse of growth cones from the temporal retina. *Neuron* 4:31-37.

Crossin, K. L., C. -M Chuong, and G. M. Edelman. 1985. Expression sequences of cell adhesion molecules. *Proc. Natl. Acad Sci. USA* 82:6942-6946.

Crossin, K. L., G. M. Edelman, and B. A. Cunningham. 1984. Mapping of three carbohydrate attachment sites in embryonic and adult forms of the neural cell adhesion molecule. *J. Cell Biol.* 99:1848-1855.

Cunningham, B. A., S. Hoffman, U. Rutishauser, J. J. Hemperly, and G. M. Edelman. 1983. Molecular topography of the neural cell adhesion molecule N-CAM: surface orientation and localization of sialic acid-rich and binding regions. *Proc. Natl. Acad. Sci. USA* 80:3116-3120.

Davies, J. A., G. M. W. Cook, C. D. Stern, and R. J. Keynes. 1990. Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones. *Neuron* 4:11-20.

Djabali, M., M. G. Mattei, C. Nguyen, D. Roux, J. Demengeot, F. Denizot, M. Moos, M. Schachner, C. Goridis, and B. R. Jordan. 1990 Aug. The gene encoding L1, a neural adhesion molecule of the immunoglobulin family, is located on the X chromosome in mouse and man. *Genomics* 7:587-93.

Doolittle, R. F. 1985. The geneology of some recently evolved vertebrate proteins. *Trends Biochem. Sci.* 10: 233-237.

Dreyer, W. J., and J. C. Bennett. 1965. The molecular basis of antibody formation: A paradox. *Proc. Natl. Acad. Sci. USA* 54:864-869.

Dreyer, W. J., and W. R. Gray. 1968. On the role of nucleic acids as genes conferring precise chemospecificity to differentiated cell lines. *In* Nucleic Acids in Immunology. O. J. Plescia and W. Braun, eds. Springer-Verlag, New York 614-643.

Edelman, G. M.and J. P. Thiery. 1990. The Cell in Contact: Adhesions and Junctions as Morphogenic Determinants. John Wiley & Sons, Inc. New York.

Edelman, G. M. 1986. Cell adhesion molecules in neural histogenesis. *Ann. Rev. Physiol.* 48:417-430.

Faissner, A., and J. Kruse. 1990. J1/Tenascin is a repulsive substrate for central nervous system neurons. *Neuron* 5:627-637.

Fischer, G., V. Künemund, and M. Schachner. 1986 Feb. Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell surface glycoprotein L1. *J Neurosci.* 6:605-612.

Fraser, S. E. 1980. A differential adhesion approach to the patterning of nerve connections. *Dev. Bio.* 79:453-464.

Fraser, S. E., and R. K. Hunt. 1978. Neuroplasticity in *Xenopus. Biophys. J.* 21:110a.

Fraser, S. E., and R. K. Hunt. 1980. Retinotectal specificity: Models and experiments in search of a mapping function. *A. Rev. Neurosci.* 3:319-352.

Furley, A. J., S. B. Morton, D. Manalo, D. Karagogeos, J. Dodd, and T. M. Jessel. 1990. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite-promoting activity. *Cell* 61:157-170.

Gennarini, G., G. Cibelli, G. Rougon, M. -G Mattei, and C. Goridis. 1989. The mouse neuronal cell surface protein F3: a phophatidylinositol-anchored member of the immunoglobulin superfamily related to chicken contactin. *J. Cell Biol.* 109:775-788.

Gierer, A. 1983. Model for the retino-tectal projection. *Proc. R. Soc. Lond.* B 218:77-93.

Grumet, M., and G. M. Edelman. 1984 May. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule *J Cell Biol.* 98:1746-1756.

Grumet, M., and G. M. Edelman. 1988. Neuron-glia cell adhesion molecule interacts with neurons and astroglia via different binding mechanisms. *J. Cell Biol.* 106: 487-503.

Grumet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA* 82:8075-8079.

Grumet, M., S. Hoffman, C. -M Chuong, and G. M. Edelman. 1984a. Polypeptide components and binding functions of neuron-glia cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* 81:7989-7993.

Grumet, M., S. Hoffman, and G. M. Edelman. 1984b. Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron-neuron and neuron-glia adhesion. *Proc. Natl. Acad. Sci. USA* 81: 267-271.

Hamburger, V. 1980. S. Ramon y Cajal, R. G. Harrison, and the beginnings of neuroembryology. *Perspect. Biol. Med.* 23:600-616.

Harrelson, A. L., and C. S. Goodman. 1988. Growth cone guidance in insects: Fasciclin II is member of the immunoglobulin superfamily. *Science* 242:700-708.

Hartlieb, E., and C. A. O. Stuermer. 1989. Pathfinding and target selection of goldfish retinal axons regenerating under TTX induced impulse blockade. J. *Comp. Neurol.* 284:148-168.

Heidmann, S. R., and E. Buxbaur. 1990. Tension as a

regulator and integrator of axonal growth. *Cell Motil. Cytoskeleton* 17:6-10.

Hekmat, A., D. Bitter-Seurmann, and M. Schachner. 1990. Immunocytological localization of the highly polysialylated form of the neural cell adhesion molecule during development of the murine cerebellar cortex. *J. Comp. Neurol.* 291:458-467.

Hoffman, S., B. C. Sorkin, P. C. White, R. Brackenbury, R. Mailhammer, U. Rutishauser, B. A. Cunningham, and G. M. Edelman. 1982. Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. Biol. Chem.* 257:7720-7729.

Hogervorst, F., I. Kuikman, A. von dem Borne, and A. Sonnenberg. 1990. Cloning and sequence analysis of Beta-4 cDNA: an integrin subunit that contains a unique 118kD cytoplasmic domain *EMBO J.* 9:765-770.

Hood, L., H. V. Huang, and W. J. Dreyer. 1977. The area-code hypothesis: The immune system provides clues to understanding the genetic and molecular basis of cell recognition during development. *J. Supramol. Struct.* 7:531-559.

Horvitz, A., K. Duggan, C. Buck, M. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin--a transmembrane linkage. *Nature* 320:531-533.

Hunkapiller, T. and L. Hood. 1989. Diversity of the immunoglobulin gene superfamily. *Advances in Immunology* 44:1-63.

Hynes, R. O., ed. 1990. Fibronectins. Springer-Verlag, New York..

Inuzuka, H., S. Miyatani, and M. Takeichi. 1991. R-Cadherin: A novel Ca^{2+} -dependent cell-cell adhesion molecule expressed in the retina. *Neuron* 7:69-79.

Jaffe, S. H., D. R. Friedlander, F. Matsuzaki, K. L.

Crossin, B. A. Cunningham, and G. M. Edelman. 1990. Differential effects of the cytoplasmic domains of cell adhesion molecules on cell aggregation and sorting-out. *Proc. Natl. Acad. Sci. USA* 87:3589-3593.

Jessell, T. M. 1988. Adhesion molecules and the hierarchy of neural development. *Neuron* 1:3-13.

Jessell, T. M., M. A. Hynes, and J. Dodd. 1990. Carbohydrates and carbohydrate-binding proteins in the nervous system. *Annu. Rev. Neurosci.* 13:227-255.

Karten, H. J., K. V. Fite, and N. Brecha. 1977. Specific projection of displaced retinal ganglion cells upon the accessory optic system in the pigeon. *Proc. Natl. Acad. Sci. USA* 74:1753-1756.

Keynes, R., and G. Cook. 1990. Cell-cell repulsion: Clues from the growth cone? *Cell* 62:609-610.

Lagenaur, C., and V. Lemmon. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. USA* 84:7753-7747.

Lem, J., M. L. Applebury, J. D. Falk, J. G. Flanner, and M. Simon. 1991. Tissue-specific and developmental regulation of rod opsin chimeric genes in transgenic mice. *Neuron* 6:201-210.

Letourneau, R. C., and T. A. Shattuck. 1989. Distribution and possible interaction of actin-associated proteins and cell adhesion molecules of nerve growth cones. *Development* 105:505-519.

Levine, J. M., L. Beasley, and W. B. Stallcup. 1984. The D1.1 antigen: A cell surface marker for germinal cells of the central nervous system. *J. Neurosci.* 4:820-831.

Liesi, P., D. Dahl, and A. Vaheri. 1984. Neurons cultured from developing rat brain attach and spread preferentially to laminin. *J. Neurosci. Res.* 11:241-251.

McCaffery, P., R. L. Neve, and U. C. Dräger. 1990. A dorsoventral asymmetry in the embryonic retina defined by protein conformation. *Proc. Natl. Acad. Sci. USA* 87:

McCaffery, P., P. Tempst, G. Lara, and U. C. Dräger. 1991. Aldehyde dehydrogenase is a positional marker in the retina. *Development* 112:693-702.

McLoon, S. 1991. A monoclonal antibody that distinguishes between temporal and nasal retinal axons. *J. Neurosci.* 11:1470-1477.

Mitchinson, T., and M. Kirschner. 1988. Cytoskeletal dynamics and nerve growth. *Neuron* 1:761-772.

Miura. M., M. Kobayashi, H. Asou, and K. Uyemura. 1991. Molecular cloning of cDNA encoding the rat neural cell adhesion molecule L1. *FEBS Letters* 289:91-95.

Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Fruh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* 334:701-703.

Müller, B., B. Stahl, and F. Bonhoeffer. 1990. In vitro experiments on axonal guidance and growth-cone collapse. *J. Exp. Biol.* 153:29-46.

Murray, B. A., G. C. Owens, E. A. Prediger, K. L. Crossin, B. A. Cunningham, and G. M. Edelman. 1986. Cell surface modulation of the neural cell adhesion molecule resulting from alternative mRNA splicing in a tissue-specific developmental sequence. *J Cell Biol.* 103:1431-9.

Napolitano, E. W., K. Venstrom, E. F. Wheeler, and L. F. Reichardt. 1991. Molecular cloning and characterization of B-cadherin, a novel chick cadherin. *J. Cell Biol.* 113:893-905.

Neugebauer, K. M., C. J. Emmet, K. A. Venstrom, and L. F. Reichardt. 1991. Vitronectin and thrombospondin

promote neurite outgrowth: Developmental regulation and role of integrins. *Neuron* 6:345-358.

Neugebauer, K. M., K. J. Tomaselli, J. Lilien, and L. F. Reichardt. 1988. N-cadherin, NCAM, and integrins promote retina neurite outgrowth on astrocytes in vitro. *J. Cell Biol.* 107:1177-1187.

Nose, A., K. Tsuji, and M. Takeichi. 1990. Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* 61:147-155.

Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between alpha-actinin and the Beta-1 subunit in vitro. *J. Cell Biol.* 111:721-729.

Ozawa, M., H. Hoschutsky, K. Herrenknecht, and R. Kemler. 1990a. A possible new adhesive site in the cell-adhesion molecule uvomorulin. *Mech. Develop.* 33: 49-56.

Ozawa, M., M. Ringwald, and R. Kemler. 1990b. Uvomorulin-Catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc. Natl. Acad. Sci. USA* 87: 4246-4250.

Patterson, P. H. 1988. On the importance of being inhibited or saying no to growth cones. *Neuron* 1:263-267.

Pollerberg, E., K. Burridge, K. Krebs, S. Goodman, and M. Schachner. 1987. The 180 kD component of the neural cell adhesion molecule N-CAM is involved in cell-cell contacts and cytoskeleton-membrane interactions. *Cell Tissue Res.* 250:227-236.

Prediger, E. A., S. Hoffman, G. M. Edelman, and B. A. Cunningham. 1988. Four exons encode a 93bp insert in three N-CAM mRNAs specific for chick heart and skeletal muscle. *Proc. Natl. Acad. Sci. USA* 85:9616-9620.

Prince, J. T., N. Milona, and W. B. Stallcup. 1989. Characterization of a partial cDNA clone for the NILE

glycoprotein and identification of the encoded polypeptide domain. *J. Neurosci.* 9:876-883.

Rabacchi, S. A., R. L. Neve, and U. C. Dräger. 1990. A positional marker for the dorsal retina is homologous to the 68kD-laminin receptor. *Development* 109:521-531.

Ramon y Cajal, S. 1960. Studies on vertebrate neurogenesis. Translated by L. Guth. 1929 reprint. C. C. Thomas, Springfield, Ill.

Ranscht, B. 1988. Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. *J. Cell Biol.* 107:1561-1573.

Raper, J. A., and J. P. Kapfhammer. 1990. The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. *Neuron* 4:21-29.

Rathjen, F. G., J. M. Wolff, R. Frank, F. Bonhoeffer, and U. Rutishauser. 1987a. Membrane glycoproteins involved in neurite fasciculation. *J Cell Biol.* 104:343-53.

Rathjen, F. G., J. M. Wolff, S. Chang, and J. Raper. 1990. Membrane glycoproteins involved in neurite-neurite interactions. *Acta Histochem Suppl.* 38:59-63:

Rathjen, F. G., J. M. Wolff, S. Chang, F. Bonhoeffer, and J. A. Raper. 1987b. Neurofascin: A novel chick cell-surface glycoprotein involved in neurite-neurite interactions. *Cell* 51:841-849.

Reichardt, L. F., and K. J. Tomaselli. 1991. Extracellular matrix molecules and their receptors: Functions in neural development. *Annu. Rev. Neurosci.* 14:531-570.

Reyes, A. A., S. J. Small, and R. Akeson. 1991. At least 27 alternatively spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. *Mol. Cell Biol.* 11:1654-1661.

Ruegg, M. A., W. T. Stoeckli, R. B. Lanz, P. Streit, and P. Sonderegger. 1989. A homologue of the axonally secreted protein axonin-1 is an integral membrane protein of nerve fiber tracts involved in neurite fasciculation. *J. Cell Biol.* 109:2363-2378.

Sage, H., R. B. Vernon, S. E. Funk, E. A. Everett, and J. Angello. 1989. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca^{2+}-dependent binding to the extracellular matrix. *J. Cell Biol.* 109:341-356.

Sanes, J. R., E. Engvall, R. Butkowski, and D. D. Hunter. 1990. Molecular heterogeneity of basal lamina: Isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. *J. Cell Biol.* 111:1685-01699.

Santoni, M. J., D. Barthels, G. Vopper, A. Boned, C. Goridis, and W. Wille. 1989. Differential exon usage involving an unusual splicing mechanism generates at least eight types of NCAM cDNA in mouse brain. *EMBO J.* 8:385-392.

Schlosshauer, B., U. Schwarz, and U. Rutishauser. 1984. Topological distribution of different forms of neural cell adhesion molecule in the developing chick visual system. *Nature* 304:

Schmidt, J. T., C. M. Cicerone, and S. S. Easter. 1978. Expansion of the half retinal projection to the tectum in goldfish: An electrophysiological and anatomical study. *J. Comp. Neurol.* 177:257-278.

Schmidt, J. T., and D. L. Edwards. 1983. Activity sharpens the map during the regeneration of the retinotectal projection in goldfish. *Brain Res.* 269:29-40.

Schmidt, J. T., and L. E. Eisele. 1985. Stroboscopic illumination and dark rearing block the sharpening of the regenerated retinotectal map in goldfish. *Neuroscience* 14:535-546.

Small, S. J., S. L. Haines, and R. A. Akeson. 1988. Polypeptide variation in an N-CAM extracellular immunoglobulin-like fold is developmentally regulated through alternative splicing. *Neuron* 1:1007-1017.

Sperry, R. W. 1963. Chemoaffinity in the orderly growth of nerve fiber patterns and connections *Proc. Natl. Acad. Sci USA* 50:703-710.

Sperry, R. W. 1943. Effect of 180 degree rotation of the retinal field on visuomotor coordination. *J. Exp. Zool.* 92:263-279.

Spring, J., K. Beck, and R. Chiquet-Ehrismann. 1989. Two contrary functions of Tenascin: Dissection of the active sites by recombinant tenascin fragments. *Cell* 59:325-334.

Suzuki, S., and Y. Naitoh. 1990. Amino acid sequence of a novel integrin Beta 4 subunit and primary expression of the mRNA in epithelial Cells. *EMBO J.* 9:757-764.

Suzuki, S., K. Sano, and H. Tanihara. 1991. Diversity of the cadherin family: Evidence for eight new cadherins in nervous tissue. *Cell Regulation* 2:261-270.

Takeichi, M. 1990. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* 59:237-252.

Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1451-1455.

Takeichi, M., K. Hatta, and A. Nagafuchi. 1985. Selective cell adhesion mechanisms: Role of the calcium-dependent cell adhesion system. *In* Molecular Determinants of Animal Form, G. M. Edelman, ed. Alan R. Liss, New York. 223-233.

Thanos, S., F. Bonhoeffer, and U. Rutishauser. 1984. Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection. *Proc. Natl. Acad. Sci. USA* 81:1906-1910.

Trisler, D. 1990. Cell recognition and pattern formation in the developing nervous system. *J. Exp. Biol.* 153: 11-28.

Trisler, D., M. D. Schneider, and M. Nirenberg. 1981. A topographic gradient of molecules in retina can be used to identify cell position. *Proc. Natl. Acad. Sci.* USA 78:2145-2149.

Vielmetter, J., J. Walter, and C. A. O. Stuermer. 1991. Regenerating retinal axons of goldfish respond to a repellent guiding component on caudal tectal membranes of adult fish and embryonic chick. *J. Comp. Neurol.* 311:321-329.

Vogel, B. E., G. Tarone, Goamcotto. F. G., J. Gailit, and E. Ruoslahti. 1990. A novel fibronectin receptor with an unexpected subunit composition ($\alpha_V\beta_1$). *J. Biol. Chem.* 265:5934-5937.

von der Marlsberg, C., and D. J. Willshaw. 1977. How to label nerve cells so that the can interconnect in an orderly fashion. *Proc. Natl. Acad. Sci. USA* 74:

Walter, J., S. Henke-Fahle, and F. Bonhoeffer. 1987a. Avoidance of posterior tectal membranes by temporal retinal axons. *Development* 101:909-913.

Walter, J., B. Kern-Veits, J. Huf, B. Stolze, and F. Bonhoeffer. 1987b. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. *Development* 101:685-696.

Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily -- domains for cell surface recognition. *Ann. Rev. Immunol.* 6:381-405.

Yoon, M. G. 1971. Reorganization of retinotectal projection following surgical operations on the optic tectum in goldfish. *Expl. Neurol.* 33:395-411.

Chapter II--A New Approach For Finding Molecules That Specify Position in the Developing Retinotectal System

Redundancy and Biological Systems

We have attempted to develop a method towards identifying new cell-surface molecules involved in the specificity of the retinotectal projection. Given some general constraints on this system, such as the strong adhesion of retinal axons on the tectum and general axon-axon repulsion, two orthogonal gradients are theoretically sufficient to encode a topographic map (Fraser and Hunt, 1980). Why then, in view of the discovery of the orthogonally graded TOP_{AP} and TOP_{DV}, would one pursue new specificity molecules?

The trivial answer is that these two molecules had not been discovered when we initiated our project, and furthermore, they have not yet been shown to function in specifying position. Antibodies against TOP_{DV} delay synapse formation but have not been shown to perturb the map.

A more general answer is that the nervous system is an extremely complex organ and is likely to rely on a number of overlapping physical, electrical and molecular systems to facilitate its development. The belief that more molecules than just TOP_{AP} and TOP_{DV} function in specifying position is supported by the existence of the other molecules discussed in chapter I. Though 10/22A8 and TRAP have unknown roles, the spatial, temporal and functional characteristics of the 33 kD growth cone collapsing molecule strongly argue for its role in labeling posterior tectum in a graded and functionally significant manner. Thus, if current thoughts on these molecules are correct, at least three molecules (TOP_{AP}, the 33 kD molecule, and the receptor for

the 33 kD molecule, and not counting molecules involved in more general adhesion, repulsion and growth promoting activities) are involved in anteroposterior specification where theoretically one, TOP_{AP}, would suffice. Therefore, we should not be surprised if many more molecules are involved.

The discovery of overlapping molecular systems to generate a robust developmental program is likely to become a common theme in biology. Historically, genes in *drosophila* were discovered by the phenotype of their mutant alleles, so the genes that were identified conformed to the rule: one gene→one polypeptide (or control region)→one function. With the use of MAb technology and the more recent enhancer trap methodology, genes are being identified in flies on the basis not of their functions but of their patterns of expression. "Redundant" genes, the loss of which are not phenotypically identifiable, have as a result been identified. For example, homologs of vertebrate cell adhesion molecules have been identified in *drosophila*, as well as novel cell adhesion molecules. Neuroglian is the *drosophila* homolog of mouse L1, a homophilic CAM implicated in neurite outgrowth, axon-axon adhesion and the migration of granule cells (Bieber et al., 1989; Moos et al., 1988). Null mutations in neuroglian show no gross defects in nervous system development, though at later stages the mutation is lethal. Similarly, null mutations in fasciclin I, a drosophila cell adhesion molecule with no relationship to vertebrate CAMs, show no gross phenotype in the CNS, and neither do mutations in the proto-oncogene Abelson tyrosine kinase. Double mutant embryos, null for both fasciclin I and Abelson tyrosine kinase, show specific defects in axon guidance during CNS morphogenesis in places where both genes are normally expressed (Elkins et al., 1990). In one defect the RP1 axon in these embryos (in 93 out of 100 embryos examined) fails to cross the midline as it would normally. Thus it appears

that neuroglian and fasciclin I each play redundant roles in neural development, a conclusion that apparently holds true in vertebrate systems as well (Neugebauer et al., 1988).

The issue of redundancy has been raised to re-emphasize the point that theoretical models of biological systems are excellent at revealing the minimal number of associations necessary to generate a working organ but are not suited to revealing the actual number of molecules involved. The coupling of fasciclin I with Abelson tyrosine kinase or the putative overlap in effects of TOP_{AP} with the Bonhoeffer molecule highlights a distinction between biological systems assembled from what is available, versus computer-simulated systems designed for their efficiency. Indeed, workers in the vertebrate visual system are daily made aware of the constraints evolutionary history can place upon development. The cell layers and fibers that relay visual information to the brain in vertebrates are found between the lens and the photoreceptor layer of the retina. This arrangement requires that optic fibers, where they exit the retina, must disrupt the photoreceptor layer at the optic fissure creating the so-called "blind spot." A less clumsy solution to the problem is seen in cephalopods whose eyes, similar in other respects to ours, arrange the ganglion cells behind the photoreceptors so that optic fibers can grow towards the brain without disrupting the visual field. The explanation for the vertebrate arrangement lies not in its efficiency or selective advantage, but in its history. The vertebrate eye descended from an ancestral structure that put nerve cell in front of, rather than behind, the pigment cell.

Evolution and the Developing Nervous System

It was with this focus on the evolutionary history of molecules that might be involved in generating neural specificity that we initiated our project. Video microscope images of growth cones in culture or of single labeled fibers *in vivo* (Stuermer, 1990; Fraser and Hunt, 1990) show filopodia extending, retracting and re-extending again in different directions responding to spatial (presumably molecular) cues. This apparent "sensing" resembles in many respects that of lymphocytes finding and invading target tissue, so nerve growth cones have been described as "T-cells on a leash.". Furthermore, it has been a long-held hypothesis in the Dreyer laboratory that molecules of the vertebrate immune response (especially immunoglobulins and the T-cell receptor) were likely to have evolved from high-specificity ligand receptors possibly used in neural development (Dreyer and Bennett, 1965; Dreyer and Gray, 1968; Hood et al., 1977).

We initiated a project aimed at identifying molecules involved in retinotectal specificity based on the assumption that they might be homologous to molecules of the vertebrate immune system. At the time, N-CAM, Ng-CAM and L1 had all been identified, but none had yet been shown to be related to the immunoglobulins (Hoffman et al., 1982; Grumet and Edelman, 1984; Rathjen and Schachner, 1984). A collaboration between the Schachner laboratory and the Dreyer group had been initiated to analyze L1 at the level of protein sequence, but this had not yet come to fruition (Faissner et al., 1985; Moos et al., 1988). Thus, in the absence of any specific information on Ig-related molecules in the nervous system (excepting Thy-1, found on neural and immune cells, and thought to be involved in homophilic binding, signal transduction and immune cell activation) (Tse et

al., 1985; Kraczek et al., 1986), general assumptions were made based upon what was known of the molecules of the immune system and the nature of the neural problem.

The immune system was the first complex cellular system to be analyzed in depth at a molecular level. Myeloma tumors secreting large quantities of cloned antibody molecules (Bence-Jones proteins) had greatly facilitated the study of these molecules. The T-cell receptor (TCR), on the other hand, had proven far more elusive than the immunoglobulins, in part because the TCR was not secreted by cloned cell lines (and in part because the TCR did not bind antigen alone). We assumed that the position-specifying molecules of the nervous system would be most similar to the T-cell receptor, and hence were likely to be:

1) Cell-surface molecules, like the TCR, and capable of transducing extracellular information into the cell.

2) Scarce, like the TCR, and as predicted by retinotectal models (Fraser and Hunt, 1980).

3) Members of large gene families, each of which shares similar size and structure, but with distinct binding specificities.

The first two simple assumptions are predicted by experiments and models of retinotectal specificity already discussed. The third assumption, that there will be a large number of related molecules, is not predicted by the models. As few as two theoretical molecules would suffice, though as discussed above, overlapping and at least partially redundant molecules may be the rule in complex systems. Recent results using PCR to clone genes based on conserved primer sequences has added weight to the notion that large gene families are common, if not ubiquitous in biology (Strathmann and Simon, 1990). Still, assumption #3 must remain unproven until the molecules specifying position are characterized and compared.

An attempt to identify these molecules based on the above assumptions is described in Chapter III. One of the guiding principals in initiating our project was that even if molecules that specified retinal position were not identified, it was likely that other important and unknown molecules would be. Chapter IV describes such a molecule, Bravo, a new member of the immunoglobulin superfamily with a novel distribution on optic fibers.

The remainder of this chapter is devoted to a brief discussion of a higher risk approach towards identifying neural positional markers that, although not pursued extensively, is instructive. This approach relies on the assumption that the neural molecules involved in specifying spatial position are homologous to the immune system receptors, Igs and the TCR. Thus, in addition to the general assumptions made above, we predicted the neural molecules would be:

4) Composed of immunoglobulin-like (V or C) domains, stabilized by intrachain disulphide bonds.

5) Disulphide bonded dimers or tetramers like the TCR and Ig respectively.

Assumption #4 dictated a number of approaches, none of which were actually attempted by me. For example, selective labeling of cysteine residues to identify Ig-like domains was not attempted because cysteines and even cysteine-rich regions are present in many non-Ig-related molecules such as the integrin β chain with its 56 cysteines (Albelda and Buck, 1990). Furthermore, approaches based on the chemical or enzymatic cleavage of Ig domains at their cysteines (to identify Ig-like molecules in the immune system) had been attempted without success in other laboratories (D. Teplow, personal communication). Finally, the faster mobility in SDS PAGE of compact and intact Ig-like domains versus reduced and unfolded

domains was noted in the experiments outlined below, but was thought too subtle a shift for accurate identification of Ig-domain containing proteins.

Assumption #5, on the other hand, proved quite tractable. In fact, one of the earliest methods for detecting the TCR relied on the then unproven assumption that like immunoglobulins, the TCR would be comprised of disulphide bonded chains. Using "diagonal gel electrophoresis," Goding (1983) was able to show that T-cells contain a disulphide dimer of two approximately 40 kD components. In a cloned T-cell line, these two chains run as single spots when analyzed by conventional 2-D electrophoresis, but the same components from mixed populations of T-cells produce highly dispersed patterns on 2D gels. Like antibody chains, these T-cell derived chains form spots on 2D gels that show singular molecular weights and heterogeneous charge composition, raising the possibility (later proven) that these disulphide bonded dimers were the TCR for antigen, or what is now referred to as the α and β chains of the TCR complex.

Diagonal Gel Electrophoresis of Neural Surface Molecules

Diagonal Gel electrophoresis combined with surface labeling of cells allows simple visualization of cell-surface disulphide bonded dimers. Since the diverse receptors of both B-cell and T-cell lineages contain interchain disulphide bridges, we postulated that the hypothetical diverse positional molecules of the developing nervous system might also contain such molecules. Utilising the T-cell receptor as a positive control, we adopted diagonal gels as a diagnostic technique in our lab.

In this technique, proteins are separated by size under non-reducing conditions in the first dimension (X, left to right), and then under reducing conditions (Y, up to down). Thus, disulphide

bonds are left intact in the first dimension and cleaved in the second. The vast majority of all surface proteins run with nearly identical mobility both before and after reduction, forming a diagonal line of X equals Y.

After ^{125}-I surface labelling of thymocytes, T-cell proteins were run on such a gel (Fig. 1). The chains of the T-cell receptor are easily visible in this autoradiogram as heterogeneous spots "below the diagonal." Other spots below the diagonal are detectable in these gels (Goding, 1983), but do not appear to show the heterogeneity of the α and β T-cell receptor chains.

This technique, we concluded, could be used in our lab to separate disulphide bonded dimers from the bulk of other proteins. As noted above, we also asked whether this technique would separate molecules with internal disulphide bonds from other molecules based on conformational changes in protein structure before and after reduction. Molecules carrying immunoglobulin domains show faster mobility under non-reducing conditions than under reducing conditions, owing to the compaction of Ig-loops when disulphides are intact. Such molecules would be expected to run above the diagonal rather than below. Examination of the thymocyte diagonal gel would be expected to reveal several such molecules but the molecular weight shifts for these conformational changes appear to be too subtle to be easily discernible in our gel system.

The diagonal gel procedure was applied to the developing retina and tectum and as other developing tissues such as the chicken wing and tail buds and drosophila imaginal disks. In most cases, "off diagonal" spots (other than those of known molecules) were not visible at all. Diagonal gels of surface components of developing chicken retina reveal only an extremely faint signal in the region expected for TCR-like molecules. The absence, or extreme scarcity, of these disulphide bonded dimers is

remarkable on developing non-immune tissue. This pilot experiment led us to the conclusion that either disulphide bonded dimers are not involved in the developing retinotectal system, or that if they are, they are present in such low amounts as to make analysis nearly impossible at this time. Furthermore, subsequent analyses of these extremely faint spots, even if possible, might ultimately reveal that these molecules have nothing to do with neural addressing and indeed are totally unrelated to the TCR. Therefore, we opted for the more conservative approach towards this problem discussed in Chapter III.

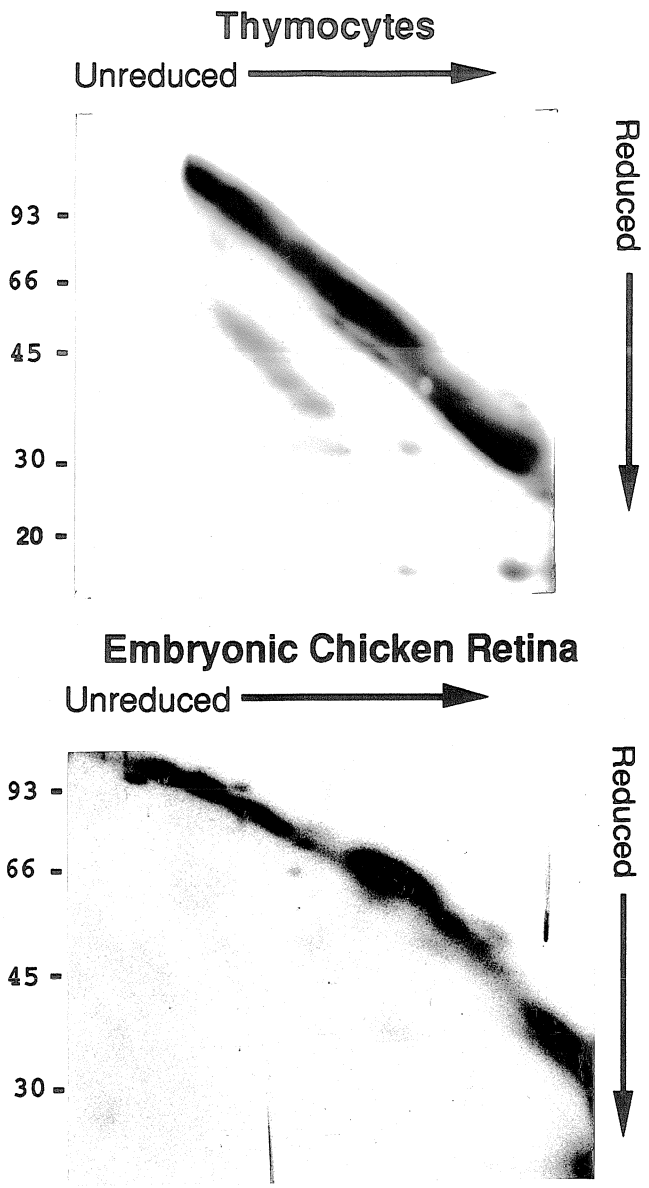

Fig 1-**Diagonal Gel Electrophoresis.** Surface components of mouse thymocytes and embryonic day 7 chicken retina were ^{125}I labeled and analyzed by diagonal gel electrophoresis. Proteins were denatured in 2% SDS in the presence of iodoacetimide to block free sulfhidryl groups and analyzed by SDS PAGE unreduced (left to right), and then reduced (with dithiothreitol) before second dimension electrophoresis (top to bottom). Molecular weight standards (reduced) are shown to left. The area enclosed by a dashed square contains a heterogeneous mix of T-cell receptor chains in the thymocyte gel, but no similarly structured molecule is detected in the retina.

References

Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB J.* 4:2868-2880.

Bieber, A. J., P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. 1989. *Drosophila* neuroglian: A member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell* 59: 447-460.

Dreyer, W. J., and J. C. Bennett. 1965. The molecular basis of antibody formation: A paradox. *Proc. Natl. Acad. Sci. USA* 54:864-869.

Dreyer, W. J., and W. R. Gray. 1968. On the role of nucleic acids as genes conferring precise chemospecificity to differentiated cell lines. *In* Nucleic Acids in Immunology. O. J. Plescia and W. Braun, eds. Springer-Verlag, New York 614-643.

Elkins, T., K. Zinn, L. McAllister, F. M. Hoffmann, and C. S. Goodman. 1990. Genetic analysis of a *drosophila* neural cell adhesion molecule: Interaction of fasciclin I and Abelson tyrosine kinase mutations. *Cell* 60:565-575.

Faissner, A., D. B. Teplow, D. Kubler, G. Keilhauer, V. Kinzel, and M. Schachner. 1985. Biosynthesis and membrane topography of the neural cell adhesion molecule L1. *EMBO J.* 4:3105-3113.

Fraser, S. E., and R. K. Hunt. 1980. Retinotectal specificity: Models and experiments in search of a mapping function. *A. Rev. Neurosci.* 3:319-352.

Goding, J. W. 1982-1983. Structure of the T-cell receptor for antigen. *In* The Walter and Eliza Hall Institute of Medical Research: Annual Review. Melbourne, Australia 51-53.

Grumet, M., and G. M. Edelman. 1984 May. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule *J Cell Biol.* 98:1746-1756.

Hoffman, S., B. C. Sorkin, P. C. White, R. Brackenbury, R. Mailhammer, U. Rutishauser, B. A. Cunningham, and G. M. Edelman. 1982. Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. Biol. Chem.* 257:7720-7729.

Hood, L., H. V. Huang, and W. J. Dreyer. 1977. The area-code hypothesis: The immune system provides clues to understanding the genetic and molecular basis of cell recognition during development. *J. Supramol. Struct.* 7:531-559.

Kraczek, R. A., K. C. Gunter, R. N. Germain, and E. M. Shevach. 1986. Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. *Nature* 322:181-184.

Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Fruh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* 334:701-703.

Neugebauer, K. M., C. J. Emmet, K. A. Venstrom, and L. F. Reichardt. 1991. Vitronectin and thrombospondin promote neurite outgrowth: Developmental regulation and role of integrins. *Neuron* 6:345-358.

Rathjen, F. G., and M. Schachner. 1984. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO J.* 3:1-10.

Strathmann, M., and M. I. Simon. 1990. G protein diversity: A distinct class of alpha subunits is present in vertebrates and invertebrates. *Proc. Natl. Acad. Sci.* USA 87:9113-9117.

Stuermer, C. A. O. 1990. Target recognition and dynamics of

axonal growth in the retinotectal system of fish. *Neurosci. Res. Suppl.* 13:S1-S10.

Tse, A. G. D., A. N. Barklay, A. Watts, and A. F. Williams. 1985. A glycophospholipid tail at the carboxyl-terminus of the Thy-1 glycoprotein of neurons and thymocytes. *Science* 230:1003-1008.

Chapter III--A Method for the Generation of Monoclonal Antibodies Against Rare Cell-Surface Molecules

Abbreviations used: Biotin-X-NHS, biotin-ε-aminocaproic acid-N-hydroxysuccinimide ester; E8, embryonic day 8; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; IPL, inner plexiform layer; KLH, keyhole limpet hemocyanin; NEPHGE, Non-equilibrium pH-gradient electrophoresis; OFL, optic fiber layer; OPL, outer plexiform layer; RT, room temperature

SUMMARY

Monoclonal antibodies (mAbs) provide a powerful tool for the identification and analysis of novel cell-surface molecules. We present here a method for antigen preparation and an immunization protocol that facilitates the generation of mAbs reactive with cell-surface molecules of low abundance and/or low antigenicity. The procedure involves isolation and extensive fractionation of cell-surface and detergent soluble extracellular matrix molecules prior to immunization. Cell-surface proteins on intact tissue are biotin labeled using a reagent that does not penetrate cells. Avidin-affinity chromatography is then used to purify these biotinylated molecules. Size exclusion high-performance liquid chromatography (HPLC) is used to separate these surface molecules on the basis of their apparent molecular weights. Finally, immunization with antigen coupled to keyhole limpet hemocyanin (KLH) is combined with long term booster immunizations to generate a hyperimmune response resulting in high-affinity IgG antibodies.

A test application of this approach was aimed at the generation of mAbs against cell-surface molecules of approximately 135 kDa in the developing chicken retinotectal system. Immunochemical analyses using antibodies produced by this approach reveal that mAbs were generated against all previously identified immunoglobulin superfamily molecules of this size in this system. Furthermore, we produced many additional antibodies that labeled retinotectal tissue in novel

staining patterns. In the two cases analyzed in detail, these new patterns reflect the distributions of previously uncharacterized members of the immunoglobulin superfamily. The success of this initial study suggests that this method represents a broadly applicable approach towards the preparation of extensive libraries of antibodies against cell-surface molecules expressed on cells from numerous sources.

INTRODUCTION

Cell-surface proteins that play critical roles in biological systems include adhesion molecules, molecules specifying cellular position and receptors of many kinds. Scientists in numerous fields such as neurobiology, developmental biology, endocrinology and cancer research have an interest in the hundreds of proteins that are displayed on cell surfaces. Although hybridoma technology has made possible the generation of monoclonal antibodies for use in the detection and analysis of specific cell-surface molecules, attempts to produce mAbs against *all* surface molecules in a given system have been hampered by the predominance of antibodies that recognize only a relatively small number of immunodominant constituents. This failure of the immune response to detect rare or poorly antigenic molecules in the presence of immunodominant ones is taken advantage of by some pathogenic microorganisms which produce highly antigenic "decoy" proteins to overload the host immune response and help the parasites evade destruction (Deans, 1984; Cully et al., 1985). Thus, a potent immune response to molecules of interest requires that the immunogen be as free as possible of other abundant or highly antigenic molecules (Benjamini et al., 1986).

Here we describe an approach to circumvent the dominant immune response to certain components and apply it to analyze the developing chicken visual system, which serves as a model

for many other systems. We have maximized the immune response to rare and previously undescribed surface molecules by extensively fractionating the proteins expressed in this system to produce an immunogen enriched for a small number of cell-surface molecules and deficient in interfering antigenic molecules. The embryonic visual system is known to utilize a sophisticated array of cell-surface molecules during the development of its complex pattern of cells and cell processes (Cowan and Hunt, 1985). Several previous attempts to identify developmentally important molecules in this system have resulted in the generation of mAbs against a small number of immunodominant molecules (reviewed in Jessell, 1988). We believe that a far more complex array of cell-surface proteins than those identified to date is required in order to facilitate the precise assembly of millions of neurons found in the visual system. Previous investigators have attempted to devise novel approaches towards the generation of a more complete library of useful mAbs. Some have utilized the immunosuppression or tolerization of mice towards undesired antigenic components prior to immunization (Hockfield, 1987; Matthew and Sandrock, 1987; McLoon, 1991; Suzue et al., 1990). Others have purified selected surface glycoproteins via interactions between their carbohydrate groups and specific lectins or antibodies (Rathjen et al., 1987). These approaches rely either on *a priori* information about the nature of molecules of interest, or on specific post-translational modifications found only on certain proteins. The approach described here differs from these in that it uses

purification of all cell-surface proteins, not just a selected subset, combined with size exclusion HPLC to fractionate the molecules prior to immunization. The results reported here confirm that this approach provides a significant step towards the generation of antibody libraries against all, even rare and poorly antigenic, cell-surface molecules. As such, it is likely to be useful in a wide variety of systems.

EXPERIMENTAL PROCEDURES

Biotin labeling

Fertilized eggs were obtained from Chino Hatcheries (Chino, CA), and eggs were incubated at 37°C until use. The surface components in intact tissue were biotinylated using the method of von Boxberg *et al.* (1990). Tissue dissected from one thousand embryos was biotinylated, twenty embryos at one time, as follows: intact tecta from twenty embryonic day eight (E8) chicken embryos were washed three times by centrifugation at 500 x g for 5 min at room temperature (RT) with 10 ml of labeling buffer (140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM $NaHCO_3$, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$. Iodoacetimide 2 mg/ml, phenylmethylsulfonyl fluoride 0.2 mg/ml and soybean trypsin inhibitor 50 µg/ml were added as protease inhibitors. The tectal pellet was resuspended in 5 ml of label buffer to which 100 µl of a freshly prepared solution of 100 mg/ml biotin-ε-aminocaproic acid-N-hydroxysuccinimide ester (Biotin-X-NHS) (Calbiochem, La Jolla, CA, USA) in dimethylsulfoxide was added, and the mixture

was shaken gently 15 min at RT. The reaction was terminated and excess reactive biotinylation reagent was quenched by washing the labeled tecta three times with 10 ml Dulbecco's Modified Eagle's Medium (Gibco).

Preparation of Antigen

Affinity chromatography with avidin was used to highly enrich surface molecules to be used as antigen for mouse immunization. Biotinylated tissue was lysed by shaking 15 min at RT in 10 ml lysis buffer: 10 mM Hepes pH 7.5, 140 mM NaCl, 4 mM EDTA, 2.5% NP40 (Sigma), 2.5% Zwittergent 3-14 (Calbiochem, La Jolla, CA), supplemented with 0.02% azide, 0.5 µl/ml aprotinin (Sigma) and the same protease inhibitor cocktail used in labeling buffer (Updyke and Nicolson, 1984). Insoluble tectal residues were removed by centrifugation at 3000 x g for 15 min at RT, and the supernate was again centrifuged 30 min at 50,000 x g at 4 °C to remove the bulk of the extracellular matrix components.

The pooled supernates from labeled and lysed tecta from 300 embryos were loaded onto a 5 ml column of avidin (monomeric)-agarose (Sigma) at 4°C at a flow rate of 30-40 ml/h. One tectum was labeled with ^{125}I using the chloramine T procedure (Greenwood et al., 1963) and added to the tectal lysate as radiometric trace. The column was washed with 100 ml of washing buffer: 0.1 M sodium phosphate buffer, pH 7.0, containing either 0.15% Zwittergent 3-14 (the detergent of choice) or 0.1% NP40. The biotinylated proteins were then eluted with 1 mg/ml D-biotin (Sigma) in washing buffer. Fractions were monitored by

UV absorbance at 260/280 nm for the presence of protein or by radiometric analysis of ^{125}I-labeled surface proteins added as trace. By this method, 5.3 mg of biotinylated proteins were purified from the tecta of 1000 embryos. The column was regenerated by washing with 200 ml of 0.1 M glycine-HCl, pH 2.0 containing 0.15% Zwittergent 3-14, followed by 200 ml of washing buffer.

The pool of biotinylated proteins (approximately 12 ml) was dialyzed against dH2O for 2 h at RT and concentrated in the dialysis tube to a final volume of 600 µl by extracting water with dry Sepharose G-75 (LKB, Sweden) placed outside the dialysis tube. The biotinylated proteins were then fractionated on the basis of their molecular weight using a TSK G-4000 SW size exclusion HPLC column (LKB, Sweden) in 0.1 M sodium phosphate buffer pH 7.0 containing 0.1% SDS at a flow rate of 0.4 ml/min. The eluate was collected in 12 fractions spanning the entire molecular weight range. 50 µg of the total protein in each fraction was coupled to KLH (Calbiochem, La Jolla, CA, USA) using a water soluble carbodiimide as follows: 25 µg of KLH was added to 50µg aliquots of each of twelve protein fractions; 20 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 50 µl of dH2O was added, and the mixture was agitated for 1 h at RT. Successful conjugation was indicated by the appearance of a visible precipitate in most fractions, including the 135 kDa fraction used in this study (Goodfriend et al., 1964).

Hybridoma Production

Antibodies were raised in Robertsonian 8.12 female mice (Jackson Labs). These mice carry the genes for the immunoglobulin heavy chains and the adenosine phosphoribosyltransferase gene on the same chromosome. Maintenance of the heavy chain genes, and thus an increase in the probability of producing antibody, is selected for in hybridomas that survive aminopterin poisoning (Taggart and Samloff, 1982). Initial immunizations were performed by emulsifying the KLH-coupled antigen with Freund's Complete Adjuvant (Sigma) and injecting this into anesthetized mice intraperitoneally and subcutaneously as well as in the footpads and tail, promoting release of the antigen over an extended period of time. Three subsequent booster immunizations were given with approximately 20 μg uncoupled antigen emulsified in Freund's Incomplete Adjuvant (Sigma) in the same locations (except the foot pads) at 3 month intervals. Four and two days before fusions were performed, the mice were boosted with approximately 20 μg of unmodified antigen injected directly into the spleen. The total elapsed time between initial immunization and final boost was 16 months, with approximately 150 μg of tectal surface protein injected per mouse.

Fusions were performed with spleen cells from the Robertsonian (8.12) mice and FOX-NY cells using the method described by Taggart and Samloff (1982). The hybridoma

supernates from cultures that showed good cellular growth were screened for reactivity on tissue (see below). Hybridomas showing spatially restricted patterns of antibody staining were frozen. Selected hybridomas were subcloned at least three times, and in many cases ascites fluid was prepared (Mishell and Shiigi, 1980).

Immunohistochemistry

Retinotectal tissue was frozen in Tissue-Tek (Miles, Elkhart, IN, USA) and sliced horizontally into 12µm sections with a cryomicrotome after tissue fixation. E8 chick embryos were fixed by overnight immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0, and then soaked in 25% sucrose for 4 h. Sections were mounted on glass slides first coated with 0.5% gelatin (Sigma) in 0.05% Cr $K(SO_4)_2$ and left to dry overnight. Sections were stained for 1 h at RT using a 1:1500 dilution of mAb ascites fluid in PBS with 10% fetal calf serum (Gibco) or using undiluted culture supernatant fluid. Sections were washed three times in PBS for 5 min at RT, followed by a 1 h incubation with FITC-conjugated goat anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:200 in PBS. Sections were washed again as before and post-fixed by incubation with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0 for 2 min at RT. Stained sections were mounted for epifluorescence in glycerol, stored at -20°C and finally photographed with a Zeiss Photomicroscope.

Gel Electrophoresis

For one dimensional analysis of size-fractionated cell-surface molecules to be used as immunogen, proteins were separated on SDS PAGE (7.5% acrylamide) using standard methods (Laemmli, 1970) and silver stained using the method of Ansorge (1985). Molecular weights were estimated by comparison with molecular weight standards from Bio-Rad Laboratories: 200 kDa, myosin; 116 kDa, β-galactosidase; 93 kDa, phosphorylase B; 66 kDa, bovine serum albumin; and 45 kDa, ovalbumin.

For two-dimensional gel electrophoresis of these proteins, the E8 tectal tissue was biotinylated as described above and solubilized to a final protein concentration of approximately 1 mg/ml in 8.5 M urea containing 2.5% Ampholine (pH 5-8) (Serva) and 5% β-mercaptoethanol. Microscale 2-dimensional electrophoresis was performed as described previously (von Boxberg et al., 1990). Protein samples were loaded onto 0.55 mm tube gels containing 9M urea and 2% Ampholine. Non-equilibrium pH-gradient electrophoresis (NEPHGE) was performed at 250 V for 4 h. Gels were soaked in SDS electrophoresis buffer for 3 min and loaded onto 0.1 mm thick 9% acrylamide slab gels for SDS PAGE.

Proteins were blotted onto nitrocellulose (Schleicher & Schull) and non-specific membrane sites blocked by overnight incubation with 0.5% Tween 20 (Sigma) in PBS. Total protein was visualized with colloidal-gold (Aurodye, Amersham). Selective visualization of biotinylated proteins was achieved by incubation of the blot with ^{35}S-streptavidin 1:1500 (Amersham) in PBS/0.05% Tween 80 for 2 h, followed by thorough

washing in 0.05% Tween 80 in PBS. Blots were exposed on X-ray film (Kodak XAR5) for approximately 48 h.

RESULTS AND DISCUSSION

Our aim was to generate mAbs against previously undescribed and possibly rare cell-surface molecules. In applying this procedure to molecules expressed in the developing retinotectal projection, we used tecta from chickens at E8 as a source of material. We presumed that E8 tecta would be a rich source of developmentally important cell-surface molecules because at this stage of development over one million retinal ganglion cell growth cones have entered the tectum and are seeking their targets. In addition, several cell-surface molecules thought to be involved in retinotectal development had been identified previously and would serve as positive controls for our approach.

Cell-Surface Biotinylation

The biotinylation reagent Biotin-X-NHS, a succinimide ester of biotin, was found previously (von Boxberg et al., 1990) to be excellent in the labeling and subsequent purification of cell-surface molecules. Under physiologically mild conditions, it binds free amino groups of protein lysine side chains. It does not penetrate the cell membrane, and the biotin moiety--separated from the protein by a six carbon spacer arm--interacts readily

with derivatives of avidin thus facilitating the subsequent purification and detection of biotinylated molecules

Two-dimensional gel electrophoresis of biotinylated tectal proteins was used to compare total tectal protein stained with colloidal gold (Fig. 1A) to biotin-labeled components visualized with ^{35}S-streptavidin (Fig. 1B). Identified cytoplasmic components such as actin and the tubulins were not labeled with streptavidin in these protein blots confirming that the biotinylation reagent did not penetrate the cell membrane. Moreover, a close examination of these blots reveals that many biotinylated proteins easily visualized after streptavidin labeling (Fig. 1B) are not detected by colloidal gold staining (Fig. 1A), presumably because these surface molecules represent too small a fraction of the total protein.

A characteristic feature of the biotinylated cell-surface molecules seen in streptavidin labeled gels is their appearance as discrete rows of spots in the electrofocussing dimension. This isoelectric point heterogeneity is unlikely to be a result of variable degrees of biotinylation since these patterns of spots are also seen in some non-biotinylated samples, and similar patterns are also detected when other (uncharged) labels are used (von Boxberg et al., 1990). Rather, this charge heterogeneity more likely is due to a number of causes. (i) Identical protein cores are differentially glycosylated, phosphorylated and otherwise post-translationally modified. (ii) Alternative splicing induces charge heterogeneity in particular proteins, in some cases without significantly altering molecular weight. (iii)

Several different molecules share the same molecular weight including members of closely related gene families. Using the vertebrate immune system as a model (Williams and Barclay, 1988), it seems reasonable to assume that evolutionary pressures would have resulted in many cell-surface molecules involved in neurogenesis belonging to highly polymorphic families, nearly identical in size, with some sequence and functional variability among the members. This idea is supported by recent results that show many of the proteins involved in cell-cell and cell-substratum interactions during neural development are related to each other as members of the immunoglobulin superfamily (Edelman, 1987), the integrin family (Tomaselli et al, 1987) or cadherin family (Takeichi,.1990). If large gene families of closely related cell-surface molecules exist in the system under study, then fractionation by molecular weight would tend to group family members together into the same immunogen.

Affinity Chromatography

Our primary concern during protein isolation was to establish conditions that would promote the complete solubilization of membrane proteins yet minimize the destruction of their native structural conformation. During the avidin-affinity chromatography procedure, maintenance of the native avidin protein conformation was necessary to achieve the high avidin-biotin affinities desired for protein binding. Also, maintenance of native protein conformation of the cell-surface molecules might be important in preserving conformational

epitopes for the generation of mAbs. Antibodies reactive with native proteins were desired since the mAbs would be used to label native antigens in tissue sections, to immunopurify antigens out of solution, and in some cases to attempt to perturb normal development in functional assays.

A lysis buffer containing a combination of zwitterionic and nonionic detergents in relatively high concentrations (2.5% each) had been previously shown to solubilize membranes while preserving sufficiently native protein structure to allow avidin/biotin and antibody/antigen interactions to take place in solution (Updyke and Nicolson, 1984). Once biotinylated and in solution, the cell-surface proteins were to be affinity purified, again with the intention to preserve native structure. The isolation of biotinylated proteins by avidin-affinity chromatography has been the subject of many previous investigations (e.g., Wilchek and Bayer, 1989). The strong affinity of the avidin tetramer for biotin ($Kd=10^{-15}$) has been found to negatively affect the ability to competitively release bound proteins. The binding of proteins to tetrameric avidin via two or more biotin moieties on the same protein makes such elution exceedingly difficult. We found that the use of avidin monomer in the column support, rather than the tetramer, facilitates elution by competitive exchange with biotin, allowing release of bound surface proteins under physiologically mild conditions.

To maintain the solubility of membrane proteins and glycoproteins in aqueous solution, detergent must be present,

though lower concentrations may be used than those originally necessary for membrane solubilization. Thus, once biotinylated proteins were bound to the monomeric-avidin column, the column was washed to remove non-specifically bound material and to exchange the high detergent-concentration lysis buffer with a buffer of lower detergent concentration before eluting bound protein with free D-biotin. We found that both the composition and concentration of detergent in the wash and elution buffer dramatically affected the elution profile. Therefore, we tested several detergent conditions for their affects on free biotin elution of protein from the column. The results of some of these tests are shown in Fig. 2 and reveal that the addition of 0.15% zwittergent to the sodium phosphate wash and elution buffers allows most of the protein to be eluted with D-biotin. In addition, the presence of 0.15% zwittergent in the elution buffer does not interfere with U.V. absorbtion at 280 nm and is also compatible with subsequent size fractionation by HPLC.

Size Fractionation of Cell-Surface Molecules

Size fractionation is an effective and general way to partially purify molecules from a large heterogeneous population, encouraging an immune response to less abundant or less antigenic molecules while simultaneously eliminating the response to antigens found predominantly in other size fractions. The TSK-4000SW size exclusion HPLC column was chosen for its high resolving capabilities in the 100-200 kDa range, a molecular weight range that had been shown previously to contain many

cell-surface molecules involved in cell-cell and cell-substratum interactions in the nervous system (Jessell, 1988; Tomaselli et al., 1987). Also, two-dimensional gel analyses of tectal cell-surface proteins show many groups of spots, potentially gene families, in this molecular weight range (Fig. 1B).

The TSK-4000 SW gel filtration column is based on an inert and non-adsorptive silica support which we have found produces good separation efficiency despite a large loading volume (0.5 ml.) The cell-surface molecules were subjected to HPLC in sodium phosphate buffer supplemented with 0.1% SDS, which not only reduces non-specific losses of poorly soluble proteins but also produces more ideal size separation during chromatography. SDS at this concentration has been found to be non-denaturing (Satta et al., 1984). Higher concentrations of SDS (1-2%) are generally required to denature proteins (*e.g.*, for SDS PAGE) (Laemmli, 1970), though after denaturation, proteins can be maintained in this state with lower detergent concentrations such as those used here. The elution profile of cell-surface molecules run on this column is shown in Fig. 3A. This report focusses on the peak marked by a horizontal bar in Fig. 3A, and proteins in this fraction are shown separated by SDS PAGE in Fig. 3B.

Immunization Strategy

Since the neural molecules present in our preparations might be highly conserved and rare, maximizing their immunogenicity was critical. The biotin moieties attached by six

carbon chains to the proteins probably serve as weak haptens to help break tolerance. In addition, to ensure a potent immune response, we conjugated the molecules to KLH, a large protein often used as carrier in immunizations. Subsequent booster immunizations were given without KLH to avoid extensive production of mAbs against KLH itself. Furthermore, we administered six immunizations over a sixteen month time period to facilitate clonal selection of B-cells producing high affinity IgG antibodies.

Anticipating the production of a large number of hybridomas requiring long-term maintenance and storage of numerous cell lines, we were concerned that a loss of antibody secretion might be a problem. Therefore, we chose to produce mAbs in mice that carry a specific chromosomal translocation that adjoins the Ig heavy chain genes with the gene responsible for surviving aminopterin poisoning, thus allowing HPRT selection for cells likely to secrete antibody.

mAbs Generated

Following sixteen months of booster immunizations, hybridomas were produced from a mouse injected with biotinylated surface proteins of approximately 135 kD. Hybridomas from this one fusion were distributed onto twenty 96-well plates and all supernates screened on cyrostat sections of paraformaldehyde-fixed E8 retinotectal tissue. Nearly 1000 viable hybridomas were selected, saved and analyzed based on the staining patterns of tissue sections. Hybridoma supernates from

three plates were tested for antibody secretion and anti-KLH reactivity using a dot-blot type assay. 100% of the hybridomas tested secreted antibody (288 out of 288). Hybridomas against KLH itself were not a significant occurrence; only 0.7% (2 out of 288) of those mAbs tested were KLH reactive. Several selected subcloned hybridoma lines were isotype tested and 97% of those tested (35 out of 36) were found to secrete IgG. IgGs are generally preferred for subsequent applications involving immunohistochemistry and immunoaffinity isolations, as well as immune screening of gel blots and expression vector libraries. The importance of KLH conjugation and the long term immunization in the generation of a large number of IgG type antibodies is suggested by comparison with the results from a previous study in which only twenty-two mAbs (all IgM's) were produced using the same immunogen as in this report, except KLH was not used and the duration of the immunization was only four months (unpublished results). We presume that periods of immunization shorter than the sixteen months used here may also be effective in the generation of high affinity IgG antibodies, but we have not studied this variable here.

The critical test of this approach is whether or not useful mAbs against cell-surface molecules involved in neural development were produced. Several of the groups of mAbs label tissue in patterns identical to those of known and previously described molecules. This identity between some of our mAbs and those of other laboratories has been confirmed by immunocrossreactivity studies as well as by comparisons of N-terminal amino acid and, in some cases, cDNA sequences (Table 1).

Thus, suggestive of the effectiveness of this approach, we have identified all of the previously described neural Ig superfamily molecules of approximately 135 kD known to us.

Table 1. *mAbs reactive with known neural proteins.*

mAb	ANTIGEN MWT (Primary Band)	EQUIVALENT MOLECULE(S)	REFERENCE
1E12	135 kDa	Ng-CAM G4 8D9	Grumet et al., 1984 Rathjen et al., 1987a Lagenaur and Lemmon, 1987
2B3	140/130 kDa	Bravo Nr-CAM	de la Rosa et al., 1990 Grumet et al., 1991
61H2	160/130 kDa	Neurofascin	Rathjen et al., 1987b
62G2	135 kDa	F11 Contactin	Brümmendorf et al., 1989 Ranscht, 1988
64E6	130 kDa	Axonin-1	Ruegg et al., 1989

In all cases, putative identities were based initially on similar patterns of retinotectal tissue staining and subsequently confirmed by various techniques including: immunocrossreactivity of purified antigens with antibodies against known neural proteins (1E12, 61H2); amino-terminal protein sequence analysis and comparison (1E12, 2B3, 62G2); cDNA sequence determination and comparison (2B3, 62G2). The assumed identity between 64E6 and axonin-1 is based only on similar patterns of staining of E8 retina, optic nerve and tectum.

In addition, we have identified twenty novel patterns of reactivity with these mAbs. Selected patterns of antibody staining of the E8 central retina, optic nerve exit and optic chiasm are shown in Fig. 4. Most of the antibodies label at least the optic fiber layer of the retina, which is not surprising given that the only component of the retina included in the immunogen was optic fibers growing on the tectum at the time of dissection. The labeling of other retinal layers, the distribution of this labeling (central vs. peripheral, nasal vs. temporal) and the apparent texture of the staining varies widely. In some cases the antibodies show strikingly restricted staining patterns. In the two such cases which we analyzed at a molecular level, these novel staining patterns reflected the distribution of two previously uncharacterized cell-surface molecules, Bravo (de la Rosa et al., 1990) and the 10/22A8 antigen (Vielmetter et al., 1991). Both of these molecules appear to be members of the immunoglobulin superfamily. Investigations of these mAbs, and others from this fusion and different molecular weight immunogens, will form the basis for future work in our laboratories.

In conclusion, the two-dimensional gel pattern of the numerous biotinylated molecules shown in Fig 1B suggests that there are many cell surface and matrix molecules in the E8 chicken tectum which have not yet been identified. The procedure described here provides a practical means of producing mAbs to these molecules. Furthermore, the general nature of this approach and its suitability for further modification (such as

with the additional use of ion exchange or reversed phase HPLC) suggests it may represent a broadly applicable approach to the preparation of extensive libraries of mAbs against cell-surface molecules and gene families in other systems as well.

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References

Ansorge, W. (1985) Fast and sensitive detection of protein and DNA bands by treatment with potassium permanganate, *J. Biochem. Biophys. Methods.* 11, 13-20.

Benjamini, E., Wan, A. M., Langton, B. C. & Andria, M. L. (1986) Cell activation and immunogenicity, *Dev. Biol. Stand.* 63, 29-39.

Brümmendorf, T., Wolff, J. M., Frank, R. & Rathjen, F. G. (1989) Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains, *Neuron* 2, 1351-1361.

Cowan, W. M.& Hunt, R. K. (1985) The development of the retinotectal projection: An Overview, in *Molecular bases of neural development* (Edelman, G. M., Gall, W. E. & Cowan, W. M., eds) pp. 389-428, John Wiley and Sons, New York.

Cully, D. F., Ip, H. S. & Cross, G. A. (1985) Coordinate transcription of variant surface glycoprotein genes and an expression site associated gene family in *Trypanosoma brucei, Cell* 42, 173-182.

Deans, J. A. (1984) Protective antigens of bloodstage *Plasmodium knowlesi* parasites, *Philos. Trans. R. Soc. Lond. (Biol.).* 307, 159-169.

de la Rosa, E. J., Kayyem, J. F., Roman, J. M., Stierhof, Y.-D., Dreyer, W. J. & Schwarz, U. (1990) Topologically restricted appearance in the developing chick retinotectal system of Bravo, a neural surface protein: Experimental modulation by environmental cues, *J. Cell Biol.* 111, 3087-3096.

Edelman, G. M. (1987) CAMs and Igs: cell adhesion and the evolutionary origins of immunity, *Immunol. Rev.* 100, 11-45.

Goodfriend, T. L., Levine, L. & Fasman, G. D.(1964) Antibodies to bradykinin and angiotensin: A use of carbodiimides in immunology, *Science.* 144, 1344-1346.

Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity, *Biochem. J.* 89, 114-123.

Grumet, M., Hoffman, S., Chuong, C. -M & Edelman, G. M. (1984) Polypeptide components and binding functions of neuron-glia cell adhesion molecules, *Proc. Natl. Acad. Sci. USA.* 81, 7989-7993.

Grumet, M., Mauro, V., Burgoon, M. P., Edelman, G. M. & Cunningham, B. A. (1991) Structure of a new nervous system glycoprotein, Nr-CAM, and its relationship to subgroups of neural cell adhesion molecules, *J. Cell Biol.* 113, 1399-1412.

Hockfield, S. (1987) A mAb to a unique cerebellar neuron generated by immunosuppression and rapid immunization, *Science* 237, 67-70.

Jessell, T. M. (1988) Adhesion molecules and the hierarchy of neural development, *Neuron.* 1,3-13.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227, 680-685.

Lagenaur, C. & Lemmon, V. (1987) An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension, *Proc. Natl. Acad. Sci. USA.* 84, 7753-7757.

Matthew, W. D. & Sandrock,A. W. (1987) Cyclophosphamide treatment used to manipulate the immune response for the production of monoclonal antibodies, *J. Immunol. Meth.* 100, 73-82.

McLoon, S. C. (1991) A monoclonal antibody that distinguishes between temporal and nasal retinal axons, *J. Neurosci.* 11, 1470-1477.

Mishell, B. B. & Shiigi, S. M. (1980) *Selected Methods in Cellular Immunology,* W. H. Freeman and Co., New York.

Ranscht, B. (1988) Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system, *J. Cell Biol.* 107, 1561-1573.

Rathjen, F. G., Wolff, J. M., Frank, R., Bonhoeffer, F. & Rutishauser, U.(1987a) Membrane glycoproteins involved in neurite fasciculation, *J. Cell Biol.* 104, 343-353.

Rathjen, F. G., Wolff, J. M., Chang, S., Bonhoeffer, F. & Raper, J. A. (1987b) Neurofascin: A novel chick cell-surface glycoprotein involved in neurite-neurite interactions, *Cell* 51, 841-849.

Ruegg, M. A., Stoeckli, E. T., Kuhn, T. B., Heller, M., Zuellig, R. & Sonderegger, P. (1989) Purification of axonin-1, a protein that is secreted from axons during neurogenesis, *EMBO J.* 8, 55-63.

Satta, D., Schapira, G., Chafey, P., Righetti, P. G. & Wahrmann, J. P. (1984) Solubilization of plasma membranes in anionic, non-ionic and zwitterionic surfactants for iso-dalt analysis: A critical evaluation, *J. Chromatogr.* 299, 57-72.

Suzue, T., Kaprielian, Z. & Patterson, P. H. (1990) A monoclonal antibody that defines rostrocaudal gradients in the mammalian nervous system, *Neuron* 5, 421-431.

Taggart, R. T. & Samloff, I. M. (1982) Stable antibody-producing murine hybridomas, *Science* 219, 1228-1230.

Takeichi, M. (1990) Cadherins: A molecular family important in selective cell-cell adhesion, *Annu. Rev. Biochem.* 59, 237-252.

Tomaselli, K. J., Damsky, C. H. & Reichardt, L. F. (1987) Interactions of a neuronal cell-line (PC12) with laminin, collagen-IV, and fibronectin- Identification of integrin-related glycoproteins involved in attachment and process outgrowth, *J. Cell Biol.* 105, 2347-2358.

Updyke, T. V.& Nicolson, G. L. (1984) Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices, *J. Immunol. Methods.* 73, 83-95.

Vielmetter, J., Kayyem, J. F., Lane, R., Roman, J., Schwarz, U. & Dreyer, W. J. (1991) A retinal cell-surface protein unequally distributed on optic fibers, *Soc. Neurosci. Abstr.* 17, 573.

von Boxberg, Y., Wütz, R. & Schwarz, U. (1990) Use of the biotin-avidin system for the labelling, isolation and characterization of neural cell surface proteins, *Eur. J. Biochem.* 190, 249-256.

Wilchek, M. & Bayer, E. A. (1989) Avidin-biotin technology ten years on: Has it lived up to its expectations? *Trends Biochem. Sci.* 14, 408-412.

Williams, A. F.& Barclay, A. N. (1988) The immunoglobulin superfamily- domains for cell surface recognition, *Annu. Rev. Immunol.* 6, 381-405.

Figures and Figure Legends

Fig. 1. *Biotinylated cell-surface proteins compared with total protein using 2-dimensional gel electrophoresis.* An E8 tectum was biotinylated and subjected to NEPHGE in the first dimension (right to left) and SDS PAGE in the second dimension (top to bottom) and blotted onto nitrocellulose membrane as described in Experimental Procedures. (A) Total protein pattern is revealed by staining of the membrane with colloidal gold. The positions of actin (a) and tubulins (t) are marked as reference points. (B) Autoradiograph of the ^{35}S-streptavidin labeled blot reveals the pattern of biotinylated surface proteins. The arrow points to the approximately 135 kDa molecules used for the immunizations described here. Possibly due to extensive glycosylation, these spots are not completely resolved by this 2-dimensional electrophoresis.

Fig. 2. *Avidin-affinity chromatography of biotinylated proteins.* Tecta were labeled with Biotin-X-NHS, lysed, and the soluble components loaded onto a 5 ml monomeric-avidin agarose column as described in Experimental Procedures. Biotinylated components were eluted with D-biotin in sodium phosphate buffer followed by column regeneration with 0.1M glycine at pH 2.0. Different detergents and concentrations were tested for their effects on elution with free biotin. For example in (A), 0.1% NP40 in the sodium phosphate buffer wash and biotin elution resulted in a small proportion of the bound material being

eluted with D-biotin and the majority being released at pH2. The detergent of choice, 0.15% Zwittergent (B), resulted in approximately 85% of the protein being eluted in the biotin peak. Protein concentrations were estimated by radiometric monitoring of ^{125}I labeled surface proteins added as trace.

Fig. 3. *Size fractionation of purified surface components by size exclusion HPLC.* Biotinylated surface molecules eluted from the avidin-agarose column with D-biotin were concentrated and loaded onto a TSK 4000 SW HPLC column for size fractionation. The flow was monitored in line for the presence of protein by absorbance at 280 nm (A), and fractions were collected. Molecules of approximately M_r 135 kD, denoted by a horizontal bar in (A), were analyzed by 6% SDS PAGE and visualized by silver staining in (B). Molecular weight standards (in kDa) are shown to the right. These size-fractionated cell-surface molecules were used to immunize mice for mAb production.

Fig. 4. *Embryonic Chicken retina and optic chiasm labeled with selected mAbs.* E8 chicken cryosections fixed with paraformaldehyde and fluorescence labeled with anti-NgCAM show the general labeling of optic fibers in the central retina including the optic nerve exit (left) and broad staining of fibers in the optic chiasm (right). In contrast, other mAbs label additional retinal layers as well and show more textured staining of fibers in the optic chiasm. mAb 1E12, an anti-NgCAM antibody, labels retinal fibers in the optic fiber layer (OFL), the optic nerve

(shown exiting the retina), and in the optic chiasm; mAb 2B3, an anti-Bravo antibody, labels the OFL, the inner plexiform layer (IPL) and the outer plexiform layer (OPL) as well as fibers in the optic chiasm; mAb 66F1 labels a subset of fibers in the OFL, optic nerve and optic chiasm; mAb 10/22A8, at this stage of development, labels the OFL, the IPL and the OPL, with OFL labeling mostly confined to the nasal (left) fibers. Note the highly restricted 10/22A8 labeling of fibers in the optic chiasm as well.

Figure 1

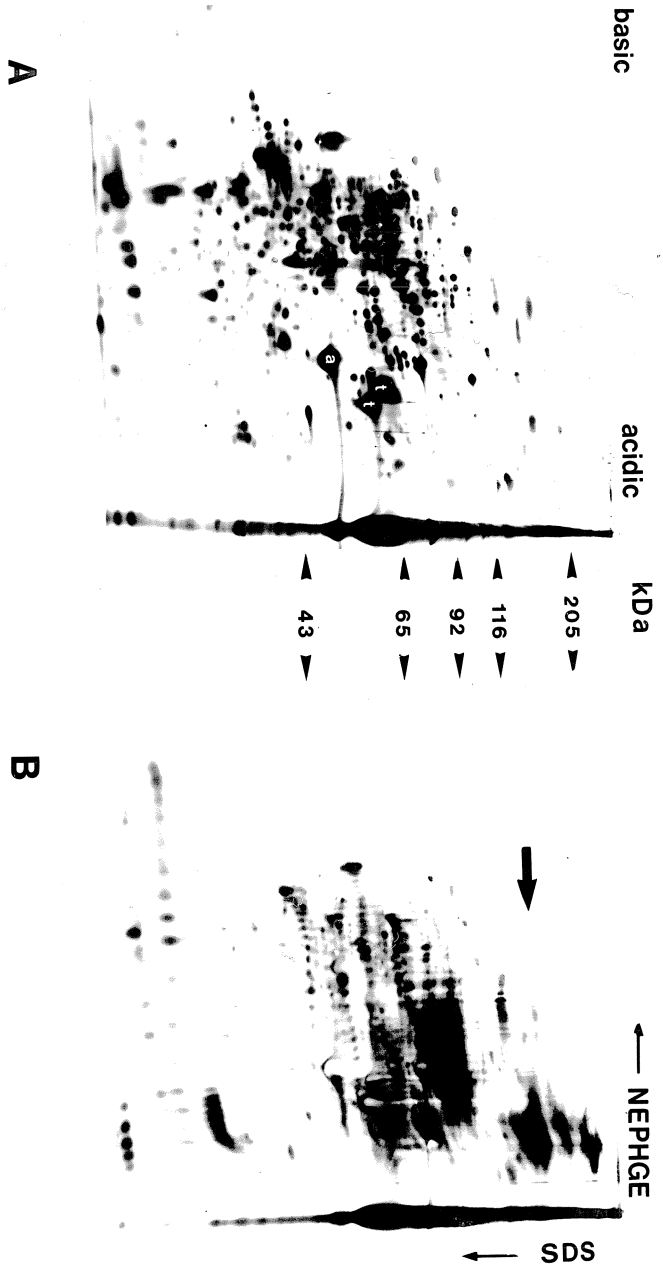

Figure 2

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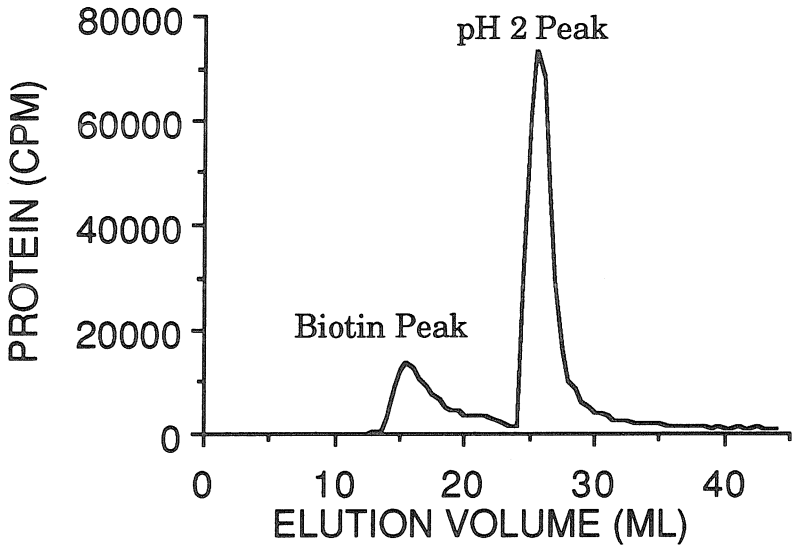
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B

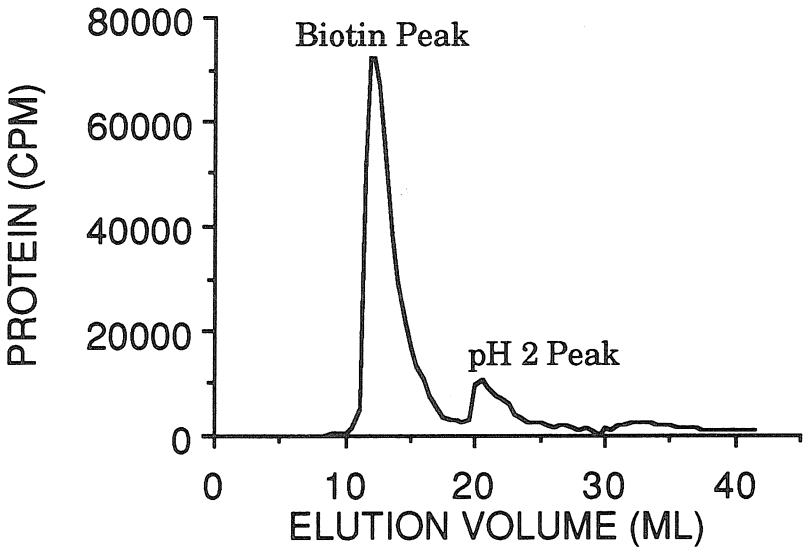
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Figure 3

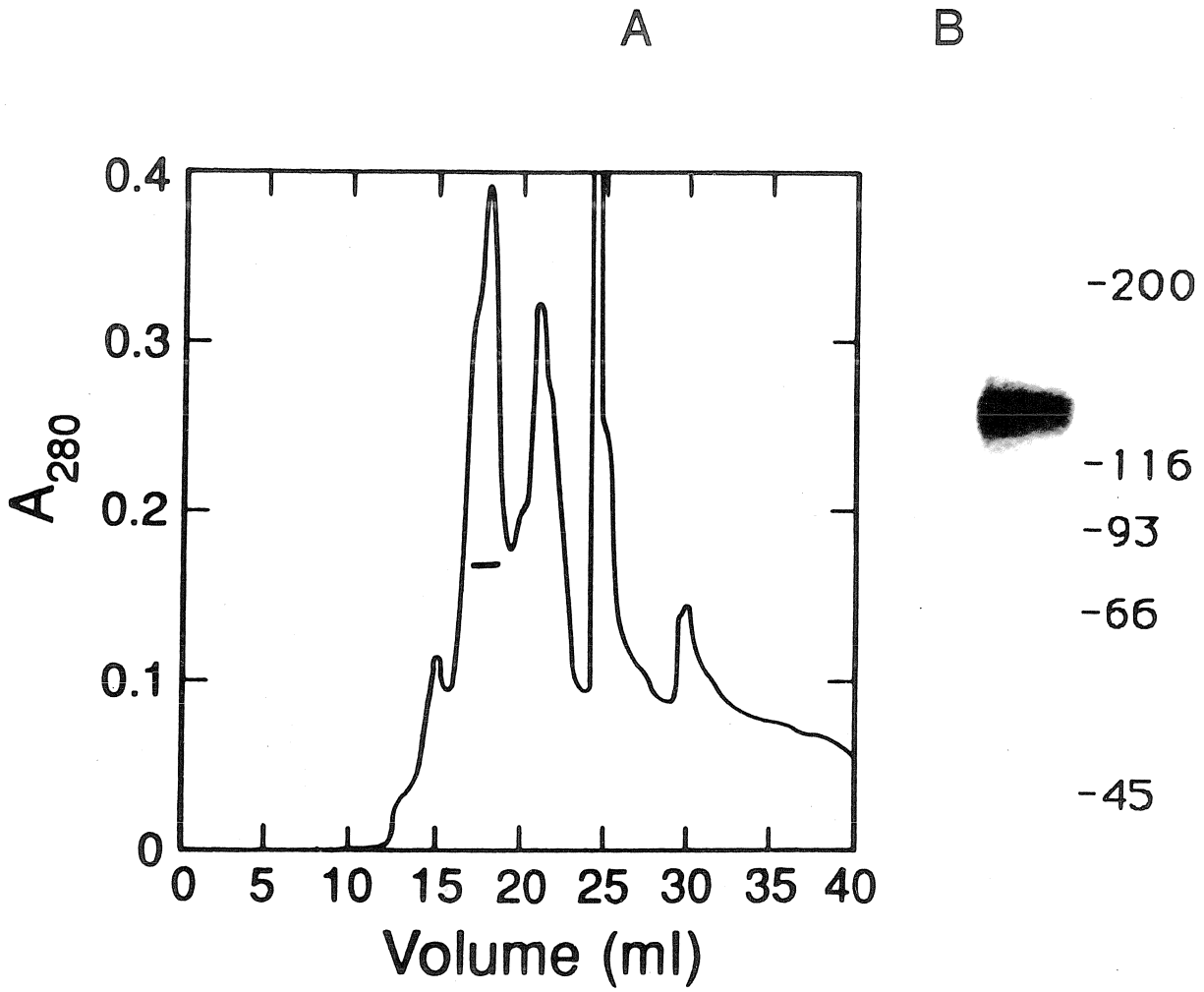
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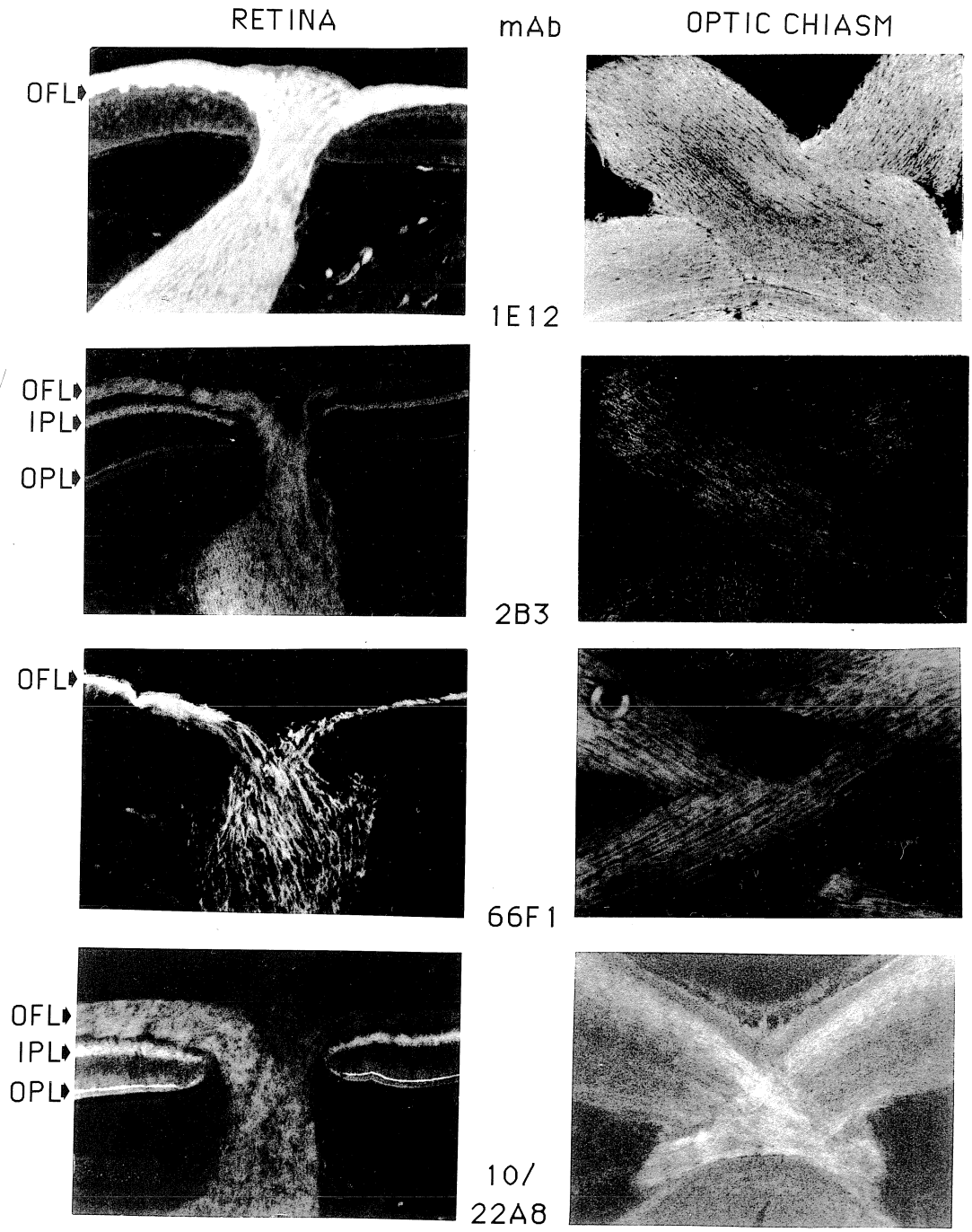
Figure 4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CHAPTER IV--BRAVO, A NEURAL MEMBER OF THE IG SUPERFAMILY, IS CLOSELY RELATED TO THE CELL ADHESION MOLECULES L1 AND NG-CAM WITH A SIMILAR HETERODIMER STRUCTURE

Abbreviations used in this chapter: ECM, extracellular matrix; E(7), embryonic day 7; FNIII, fibronectin type III homology repeat; CAM, cell adhesion molecule; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM; Nr-CAM, Ng-CAM related CAM; OFL, optic fiber layer; PCR, polymerase chain reaction; PFA, paraformaldehyde; RT, room temperature.

Abstract. Diverse cell-surface molecules of the nervous system play an important role in specifying cell interactions during development. Using a method designed to generate monoclonal antibodies (mAbs) against neural surface molecules of defined molecular weight, we have previously reported on the surface protein, Bravo, found in the developing avian retinotectal system. Bravo is immunologically detected on developing optic fibers in the retina, but absent from distal regions of the same fibers in the tectum. Here we show, in addition, that Bravo is found on Müller glial processes and end-feet and in the inner and outer plexiform layers of the developing retina.

We have isolated cDNA clones encompassing the entire coding region of Bravo, including five alternative sequences of cDNA found in some Bravo clones but absent from others. These putative alternatively spliced sequences encode stretches of polypeptide ranging in length from ten to ninety-three amino acids and are predicted to be both extra- and intracellular. The deduced primary structure of Bravo reveals that, like the cell adhesion molecules (CAMs) chicken Ng-CAM and mouse L1, Bravo is composed of six Ig-like domains, five fibronectin type III repeats, a transmembrane domain and a short cytoplasmic region. Recently, the cDNA sequence of a related molecule, Nr-CAM, was reported and its possible identity with Bravo discussed (Grumet, M., V. Mauro, M. P. Burgoon, G. E. Edelman, and B. A. Cunningham. 1991. *J. Cell. Biol.* 113:1399-1412). Here we confirm this identity and moreover show that Bravo is a heterodimer structure, composed of an α chain of M_r 140/130 and a β chain of 60-80 kD. As with L1 and Ng-CAM, the two chains of Bravo are generated from an intact polypeptide by cleavage at identical locations and conserved sites within all three molecules (Ser-Arg/Lys-Arg). The similar domain composition and heterodimer structure, as well as the 40% amino acid sequence identity of these molecules, defines them as an evolutionarily related subgroup of Ig-like CAMs. The relationship of Bravo to molecules known to be involved in cell adhesion and process outgrowth, combined with its pattern of expression and numerous potential isoforms, suggests a complex role for this molecule in cell interactions during neural development.

Introduction

The development of a functioning nervous system involves the creation of a complex network of cells and cell processes. In vertebrates, this often requires axons born in one area to project and form synapses in distant areas of the nervous system. The visual system of lower vertebrates is particularly well-suited to studies of patterned neuronal projections. In general, fibers from retinal ganglion cells grow towards the optic fissure, exiting the retina as the optic nerve which crosses the chiasm and innervates the optic tectum. A topographic map of the retina is formed on the tectum with neighboring retinal cells projecting to neighboring tectal cells.

The retinotectal system lends itself well to molecular analysis because cells and cell processes in this system are known to interact with each other and with components of the extracellular matrix (ECM) via a wide variety of molecules. An increasing body of primary structure data has revealed that, in general, these cell-surface and ECM molecules are "mosaic" proteins (Doolitle, 1985) assembled--presumably by exon shuffling--from a number of different structural units or domains (see reviews in Bork, 1991). The cadherins, for example, are integral membrane glycoproteins that promote homophilic cell adhesion in a calcium-dependent manner, especially in the early development of the nervous system (Takeichi, 1991; Inuzaka et al., 1991). Members of the cadherin superfamily, which include some desmosomal glycoproteins such as the desmocollins and desmoglein (Magee and Buxton, 1991), are composed of four repeated external domains, as well as a transmembrane region and a highly conserved cytoplasmic domain thought to interact with the cytoskeleton (Takeichi, 1990).

Similarly, the integrins are a developmentally regulated family of integral membrane receptors for ECM components such as laminin and fibronectin (Hynes, 1987; Reichardt and Tomaselli, 1991). Integrins share a basic heterodimer structure, an α chain that includes several putative Ca^{++} binding regions noncovalently

associated with a β chain that contains four cysteine-rich repeats. Both chains contain transmembrane domains and short cytoplasmic sequences thought to interact with cytoskeletal actin filaments (Albelda and Buck, 1990).

The diverse molecules of the extracellular matrix such as collagen, laminin, fibrinogen, fibronectin, thrombospondin, vitronectin and von Willebrand factor (reviewed in Reichardt and Tomaselli, 1991) also demonstrate a mosaic nature--being composed of regions with extensive internal homology repeats and homologies with other known proteins (Engel, 1991). For example, the amino acid sequence of the ECM component, tenascin (also called cytotactin), reveals a region with homology to fibrinogen, several fibronectin type III repeats (FNIIIs), a series of epidermal growth factor-like repeats, several heptad repeats associated with alpha helices of ECM proteins, and one unique terminal region involved in multimeric assembly (Chiquet-Ehrismann, 1991). Alternative mRNA splicing of exons encoding specific homology units generates further diversity in these molecules. In tenascin, tissue-specific alternative mRNA splicing generates multiple isoforms that differ in the number of FNIIIs included in the protein product (Siri et al., 1991; Prieto et al., 1990) and other isoforms, J1-160 and J1-180, may also exist (Pesheva et al. 1989). Genomic analysis of the fibronectin gene has revealed that each of the seventeen FNIIIs is separated precisely from its neighbors by one intron, and the alternatively spliced FNIIIs of fibronectin are encoded by single exons. Tissue-specific alternative splicing of fibronectin generates isoforms with distinct integrin binding properties (Komoriya et al., 1991), emphasizing the potential significance of alternative mRNA processing in development.

Another important family of cell-surface molecules found in the nervous system, and immune system as well, includes molecules containing repeats of primary structures related to immunoglobulin domains. Molecules containing multiple Ig-like domains include the major receptors of the immune system (*e.g.*, immunoglobulins, the T-cell receptor, CD4, CD8, etc.) (Williams and Barclay,1988; Hunkapiller and Hood, 1989), receptors for growth factors such as

platelet-derived growth factor (PDGF) (Yarden et al., 1986), and macrophage colony-stimulating factor (Sherr et al., 1985). Also included in this immunoglobulin superfamily (Williams, 1986) are several neural cell-surface molecules involved in Ca^{++}-independent adhesion, collectively known as Ig-CAMs (Salzer and Colman, 1989).

The neural cell adhesion molecule N-CAM is the most abundant and well-studied of the Ig-CAMs. N-CAM appears early in development and is expressed through to adulthood on neurons in chicken (Edelman and Thierry, 1990). *In vitro*, it promotes homophilic cell-cell adhesion via a Ca^{++}-independent mechanism and has been proposed to play a major role in a variety of developmental events including axon-axon adhesion, neuroblast migration, stabilization of synaptic junctions and varied roles in other tissues including muscle, kidney, heart and feathers (Edelman, 1986; Crossin et al., 1985; Chuong et al., 1985). N-CAM is composed of five immunoglobulin-like domains and two FNIIIs. Alternatively processed C-terminal sequences encode either the gylcosylphosphatidylinositol (GPI) anchored form of the molecule called NCAM-120 or the integral membrane isoforms NCAM-180 or NCAM-140 (reviewed in Edelman and Crossin, 1991; Pollerberg et al., 1987).

Molecules with multiple C2 category Ig-domains and FNIIIs define an ancient family (older than the divergence of arthropods and chordates) of Ig-CAMs that includes: an insect molecule with NCAM-like structure, fasciclin II (Harrelson and Goodman, 1988); L1 found in mouse (Moos et al., 1988), in rat (Prince et al.,1989; Miura et al., 1991), in human (Kobayashi et al., 1991) and a related insect molecule, neuroglian (Bieber et al., 1989); Ng-CAM in chicken (Burgoon et al., 1991); Contactin/F11 found in chicken (Ranscht et al., 1988; Brümmendorf et al., 1989) and called F3 in mouse (Gennarini et al., 1989); and TAG-1 found in rat (Furley et al., 1990), preliminarily identified as axonin-1 in chicken (Ruegg et al., 1989). Many of these Ig-CAMs have been shown to be involved in cell-cell adhesion and axon fasciculation and in the promotion of neurite outgrowth and cell migration (Jessell, 1989). In addition, the restricted pattern of expression of some of these molecules, TAG-1

(Dodd et al., 1988), fasciclin II and neuroglian, suggest they might serve as cell or axonal guidance molecules.

In an attempt to identify additional members of the Ig-CAM family, we initiated a project aimed at generating monoclonal antibodies (mAbs) specifically against cell-surface molecules of the same molecular weight as many of these family members, approximately 135 kD. Using this approach, we identified not only all of the chicken Ig-CAMs noted above (see Chapter III), but also a number of other molecules including the novel neural cell-surface molecule Bravo.

Bravo shows a striking pattern of topological restriction in the embryonic chicken retinotectal system (de la Rosa et al., 1990). Bravo is present on the retinal portion of optic fibers during the period of time when optic fibers are growing towards the tectum and seeking their targets, but it is not detectable on tectal regions of these same optic fibers. Preliminary amino acid sequence analysis suggested Bravo is a member of the immunoglobulin superfamily most closely related to mammalian L1 and chicken Ng-CAM (de la Rosa et al., 1990; Kayyem, J. F., J. M. Roman, E. J. de la Rosa, U. Schwarz and W. J. Dreyer. 1990. *J. Neurosci. Abstracts* 16:1010).

Here we present supporting results from protein analysis and molecular cloning of Bravo cDNAs which indicate that Bravo is a new member of the immunoglobulin superfamily, related to the neural cell-surface molecule L1 (Moos et al., 1988) and related but not identical to chicken Ng-CAM (Rathjen et al., 1987; Burgoon et al., 1990). Like mouse L1, chicken Ng-CAM and drosophila neuroglian, Bravo contains six Ig domains and five FNIIIs and is an integral membrane protein. In contrast to previously reported results on the identical molecule, Nr-CAM (Grumet et al., 1991), Bravo is a heterodimer composed of an α chain of M_r 130/140 noncovalently attached to a β chain of 60-80 kD. Both L1 and Ng-CAM are also found primarily as heterodimers with comparable components of approximately 135 and 80 kD (Burgoon et al., 1990; Faissner et al., 1985). The deduced amino acid sequence of Bravo, L1 and Ng-CAM further reveals that the α and β chains of each of these molecules

are derived from intact polypeptides by proteolytic cleavage at an identical location and conserved site on each molecule.

The nature of the restricted pattern of Bravo expression as well as the sequence and structural relationship to L1 and Ng-CAM--both known to be involved in neuron-neuron adhesion, axon fasciculation and outgrowth of neurites--suggests Bravo is involved in fiber contacts and fasciculation during development. Furthermore, by sequencing five overlapping Bravo clones, we have identified five putative alternatively spliced sequences. The possible controlled splicing of these sequences, three extracellular and two intracellular, offers the intriguing possibility that differential processing of Bravo mRNA significantly increases the potential functions of this cell-surface molecule.

Materials and Methods

Immunohistochemistry

Eight anti-Bravo mAbs and one Anti Ng-CAM mAb used in these experiments were generated utilizing a novel method for isolating, fractionating and generating a vigorous murine immune response against tectal surface components of approximately M_r 135 kD (see Chapter III). Embryonic day eight (E8) chick embryos were fixed using either paraformaldehyde (PFA) or trichloroacetic acid (TCA). Tissue was immersed overnight at RT in 4% PFA or 2% TCA in 0.1 M sodium phosphate buffer, pH 7.0, and soaked in 30% sucrose for 4 hr. Retinotectal tissue was sliced horizontally into 12 µm sections using a freezing microtome, adhered to poly-lysine coated slides and allowed to air-dry overnight at RT.

Sections were stained using a 1:1500 dilution of mAb ascites fluid in PBS with 10% fetal calf serum (Gibco) or using undiluted culture supernatant fluid for 1 h at RT. Sections were washed three times in PBS for 5 min at RT, followed by a 1 h incubation with fluorescein-labeled goat anti-mouse Ig (Cappel) diluted 1:200 in PBS. Sections were washed again as before and post-fixed by

incubation with 4% PFA in 0.1 M sodium phosphate buffer, pH 7.0 for 2 min at RT. Finally, stained sections were mounted in glycerol and photographed with a Zeiss Photomicroscope.

Purification of Antigen by Immunoaffinity Chromatography

Prior to its immobilization on a column, anti-Bravo mAb 2F12 and anti-NgCAM mAb 2B8 were purified from mouse ascites by chromatography on a CM Affi-Gel Blue column (Biorad), according to the manufacturer's instructions. Purified mAb (10 mg) was bound to 3 ml of Affi-Gel 10 (Biorad) by overnight incubation with gentle agitation in PBS at 4°C as recommended by the manufacturer. A column of each solid support was pre-run with the various buffers to be used for antigen purification (see below).

Bravo and Ng-CAM proteins were purified from post-hatching day 1 (P1) chicken brains which were first washed three times for 5 min in 140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM $NaHCO_3$, 1.5 mM $MgSO_4$ and 1.5 mM $CaCl_2$ with iodoacetimide (2 mg/ml), phenylmethylsulfonyl fluoride (0.2 mg/ml) and soybean trypsin inhibitor (50 µg/ml) added as protease inhibitors. Brains were lysed by shaking tissue for 15 minutes at RT in lysis buffer (3 ml per brain): 10 mM Hepes pH 7.5, 140 mM NaCl, 4 mM EDTA, 2.5% NP40 (Sigma), 2.5% zwittergent 3-14 (Calbiochem), supplemented with 0.02% azide and 0.5 µl/ml aprotinin in addition to the same protease inhibitors used for washing (Updyke and Nicolson, 1984). After centrifugation (50,000 g, 30 minutes, 4°C) the supernate from 200 brains was gravity loaded onto the affinity column at a flow rate of approximately 30 ml/h. To remove nonspecifically and weakly bound material, the 3 ml column was washed as follows: i) 50 ml PBS containing 0.5% NP40; ii) 20 ml of 20mM Tris-HCL, pH 8.0 containing 140 mM NaCl, 0.5% NP40 and 0.5% Zwittergent 3-14; iii) 10 ml of 50 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl and 0.5% NP40; and iv) 10 ml of 50 mM Tris-HCl, pH 9.0 containing 0.5 M NaCl and 0.1% NP40. The antigen was eluted with 12 ml of 50 mM triethanolamine, pH 11.5, containing 150 mM NaCl and 0.1% NP40. Fractions of 1.2 ml were collected in tubes containing 0.3 ml of 1M Tris-HCl, pH 6.7.

The fractions were analyzed using SDS PAGE and peak fractions were concentrated in a centricon-30 (Amicon) following the manufacturers instructions. Finally, the column was regenerated by washing with 20 ml of 20 mM Tris-HCl, pH 8.0, containing 140 mM NaCl and 0.025% azide.

Protein Analysis

Purified proteins were separated on SDS PAGE (6% or 7.5% acrylamide) using standard methods (Laemmli, 1970) and silver stained using the method of Ansorge (1985). Dithiothreitol (75 mM) or iodoacetimide (100mM) was added to the protein samples before analyses by SDS PAGE to produce reducing or nonreducing conditions respectively. Molecular weights were determined by comparison with molecular weight standard from Bio-Rad Laboratories: 200 kD, myosin; 116 kD, β-galactosidase; 93 kD, phosphorylase B; 66 kD, BSA; and 45 kD, ovalbumin.

For Western blots, proteins were transferred to Hybond-C (Amersham) using essentially the method of Towbin et al., 1979. After blocking sites on the membrane overnight with 5% BSA in PBS, the membranes were labeled with mAb ascites fluid at a 1/1000 dilution for 1 h, or with a 1/20 dilution of the anti-HNK-1 mAb "Anti-Leu 7" from Beckton-Dickenson. Reactive proteins were subsequently visualized using standard biotin/avidin sandwich methods with biotinylated goat anti-mouse immunoglobulin (Amersham, 1: 200) and HRP conjugated streptavidin (Amersham, 1:200) visualized with 4-chloronapthol (0.5 mg/ml) and H_2O_2 (0.015%) in PBS.

Amino-terminal and internal protein sequence information was gathered using the Applied Biosystems Model 477A protein sequenator with on-line PTH analysis (A.B.I. Model 120A). Antigens (30-200 pmole) were purified by affinity chromatography as described above, and subsequently electroblotted onto polyvinylidene difluoride (PVDF) membrane following SDS-PAGE (Matsudaira, 1987). Alternatively, peptides were generated from the affinity purified proteins by treatment with V8 protease (Sigma) overnight at 37°C at

a protein/enzyme ratio of 50/1 (wt/wt), before separation on a C18 (Pharmacia) HPLC column (Faissner et al., 1985). Peak fractions were collected while monitoring absorbance at 215 nm, then loaded directly onto the protein sequenator.

DNA Cloning and Sequencing

Total RNA was prepared from E7 retina and from E17 cerebella by homogenization of tissue in cessium trifluoracetic acid (Carter et al., 1983). Poly (A)+ RNA was prepared using oligo (dT) spin columns from Pharmacia. cDNA libraries were constructed in λZapII (Stratagene) from the poly (A)+ retinal RNA using the RNAse H method (Gubler and Hoffman, 1983) and random oligonucleotides (Promega) as primers. A library was also prepared by Stratagene from the poly (A)+ cerebellar RNA using random oligonucleotides as primers.

All three libraries were screened with a mixture of eight anti-Bravo mAbs: 2B3, 2F12, 3A10, 1C10, 3E11, 3G9, 1C4, 1F3 (Young and Davis, 1983). The mAbs were pooled and diluted in PBS such that each mAb was represented at 1/1000th the concentration of its ascites fluid. Five putatively Bravo plaques were identified using HRP-conjugated anti-mouse Ig. Only one of these (from the Stratagene E17 cerebellar poly (A)$^+$ library) was reactive with Bravo mAbs after plaque purification to homogeneity (Maniatis et al., 1982).

This clone, Bravo-1, was subcloned into the plasmid pBluescript using an *in vivo* excision technique as per Stratagene's instructions. The cDNA was partially sequenced from pBluescript using dideoxy sequencing and Sequenase 2 (U.S. Biochem) (Sanger et al., 1977), and its identity with Bravo confirmed by exact matching at eighteen out of eighteen residues of the cDNA derived sequence with amino acid sequence data generated from a Bravo peptide. After EcoRI excision of the Bravo-1 insert and subsequent subcloning into Pmob vector (kindly supplied by Dr. Michael Strathmann), the remainder of Bravo-1 was sequenced using a

transposon based strategy (Strathmann et al., 1991)

To identify remaining Bravo cDNA sequences, Bravo-1 insert was labeled with ^{32}P-dCTP by random oligonucleotide priming (Feinberg and Vogelstein, 1984) and used to screen the cerebellar cDNA library. Oligonucleotide primers (Caltech Microchemical Facility) matching either the 5' or the 3' end of the Bravo-1 insert were used in conjunction with vector primers (M13 universal and reverse primers) in PCR reactions to determine which of the positive clones contained the most new 5' and 3' sequences respectively. N1 and C1 were derived in this way (Fig. 6).

For sequencing, N1 and C1 were purified to homogeneity and subcloned into pBluescript as before. Synthetic oligonucleotide primers were used in both the sense and anti-sense directions to determine the new sequences (with some overlap with Bravo-1) of these clones. To identify sequences encoding the C-terminus of Bravo, the 3' end of C1 was amplified and labeled by PCR. Using primers spaced 300 bp apart, a band was PCR amplified from C1 DNA and gel purified using standard protocols. The purified fragment was then labeled using the "single-stranded PCR" technique by amplification through 35 linear cycles with the sense PCR primer incorporating ^{32}P-dCTP as the only source of cytosine. This single-stranded probe was used to screen the cerebellar cDNA library, and positive clones were mapped by PCR. Two clones, C2 and C3, encoding alternative C-termini of the protein were identified in this way, and sequenced as described above.

cDNA sequences were compiled using the GCG system software package (Devereux et al., 1984). Translated sequences from Bravo and from the Genbank database were compared pairwise using the FASTA algorithm (Pearson and Lipman, 1988) to determine percent sequence identities.

Results

mAbs raised against E8 tectal surface proteins of approximately 135 kD stained retinotectal tissue sections in a variety of patterns. At least nineteen patterns of reactivity have been identified from mAbs of this fusion, including a group of mAbs we originally called Alpha (using the international phonetic alphabet). The Alpha staining pattern is identical to that of mAbs against the neural cell-surface molecule Ng-CAM, which is also known as 8D9 and G4 (Grumet and Edelman, 1984; Lemmon and McLoon, 1986; Rathjen et al., 1987) (Fig. 1). Immunocrossreactivity studies on purified antigen, and N-terminal protein sequencing revealed that the Alpha antigen is identical to Ng-CAM (data not shown).

Another group of mAbs we call Bravo were found to stain tissue in a pattern similar to but distinct from Ng-CAM. In contrast to the large number (>100) of Alpha (anti-Ng-CAM) mAbs that were generated in this fusion, only eight Bravo mAbs were detected, perhaps reflecting the relative abundance or antigenicity of these two molecules. Three Bravo mAbs (2B3, 2F12 and 3E11) were shown by amino acid sequence analysis and protein immunoblotting to react with an identical protein which was further characterized and compared to Ng-CAM.

Distribution of antigen

Anti-Bravo mAbs stain all fiber layers of the retina as soon as they are morphologically identifiable; whereas Ng-CAM staining in the retina (Rathjen et al., 1987) is primarily confined to the ganglion cell fibers of the Optic Fiber Layer (OFL). At E6, anti-Bravo and anti-Ng-CAM labeling of the developing retina reveals a nearly identical pattern of staining in the OFL (though Bravo staining is always less intense than Ng-CAM). At E8, the pattern of antibody labeling is dramatically different; Bravo stains both the Inner Plexiform Layer (IPL) and the Outer Plexiform Layer (OPL), while no such Ng-CAM staining is evident (Fig. 1).

Differences in the localization of Bravo and Ng-CAM outside the retina were also found. We have previously shown that Bravo (and not Ng-CAM) labeling of optic fibers is restricted along the axon length to the proximal region of the retinal axons and absent or dramatically reduced from distal regions in the tectum (de la Rosa et al., 1990). In the developing tectum, although Bravo and Ng-CAM both label circumferential fibers, they do so with different intensities and patterns of distribution. Tectal tissue in the presumptive *Stratum opticum* (Crossland et al., 1975) is labeled by Bravo and not Ng-CAM mAbs, while the optic fibers themselves display Ng-CAM and not Bravo in these tectal regions (de la Rosa et al., 1990). Outside the retinotectal system, both Bravo and Ng-CAM appear simultaneously on fibers emerging from the early neural tube, and in the developing cerebellum, both Ng-CAM and Bravo are found primarily on fibers in the external granule cell layer (data not shown).

Bravo, but not Ng-CAM, has also been localized to glial processes. In the OFL, Müller glial processes and end-feet are lined up in parallel rows with bundles of retinal axons running between them toward the optic fissure. In most immunohistologically stained sections, dense labeling of the optic fiber fascicles (Fig. 1) would obscure any staining of the glia and their end-feet in the OFL; however, certain sections situated along rows of glial processes show a clear pattern of Bravo staining indicative of the labeling of Müller glial processes and end-feet (Fig. 2). Also, the possible labeling of ventricular cell processes and end-feet in the tectum has been implicated in the Bravo staining of tectal structures in the vicinity of the *Stratum opticum* (de la Rosa et al., 1990).

Bravo labeling of the developing retina is also distinguished from Ng-CAM labeling by variations in Bravo reactivity in response to differing tissue fixation conditions. Both Bravo and Ng-CAM label the entire OFL in tissue sectioned after TCA fixation and in unfixed cryosections as well. In contrast, when tissue is fixed with PFA, Bravo labeling appears only on the vitreal surface of the OFL (through which newly emerging optic fibers grow) and not on older fibers in deeper (less vitreal) layers of the OFL (Fig. 3). The absence

of Bravo labeling of deeper optic fibers is not an effect of differential fixative or mAb penetration, since tissue on both sides of the unlabeled deeper layers of the OFL--surface optic fibers on one side and processes in the IPL on the other--are labeled by Bravo after PFA fixation.

The different modes of action of PFA and TCA may explain the fixation-dependent staining of Bravo mAbs. PFA fixes tissue by crosslinking exposed proteins while TCA actually denatures and precipitates the proteins. The reduced staining with anti-Bravo in the OFL after PFA treatment suggests either a loss or masking of the Bravo epitope. Loss of the Bravo epitope in less vitreal layers of the OFL due to prolonged exposure to PFA is unlikely since regions equally if not more heavily exposed to the fixative are labeled by Bravo mAbs after PFA fixation. It seems more likely that the absence of Bravo staining on less vitreal and older fibers in the OFL is a result of the masking of Bravo epitopes in this region due to dense networks of protein cross-links created by PFA fixation.

This variable staining of the OFL by Bravo mAbs after PFA versus TCA fixation suggests the possibility that on older optic fibers, Bravo is more tightly associated with, and possibly covered up by, other surface molecules such as Ng-CAM. It is interesting to note that in addition to Bravo, several other groups of mAbs in our library (not including anti-NgCAM mAbs) showed the same fixation-dependent surface labeling of the OFL, implying that this putative "masking" phenomenon may be widespread and that published accounts of surface antigen restrictions (particularly when characterized only by PFA fixed histological sections) may need to be reevaluated.

Bravo Protein Characterization

Bravo mAbs were used to immunopurify antigen from different sources of chicken neural tissue to determine whether tissue-specific or developmentally-specific changes occur in the Bravo antigen. With each brain tissue tested (E8 and E12 tecta, E17 cerebella and P1 whole brain), Bravo was immunoaffinity-isolated

from the detergent-lysed tissue using an anti-Bravo solid support. The column eluates were analyzed by SDS PAGE and in each case showed a prominent doublet at 140/130 kD, as well as a diffusely migrating set of bands centered around 60 kD and 80 kD (Fig. 4). Thus, no obvious differences exist in the pattern of the SDS gel bands of Bravo isolated from these different tissues.

This pattern of bands on SDS PAGE is nearly identical to the patterns found with mouse L1 (Sadoul et al., 1988) and with chicken Ng-CAM (Rathjen et al., 1987)--shown here using Alpha mAb, 1E12 (Fig. 4, lane 1). The major components purified by the anti-L1 mAb are a 140 kD band and a broad band of approximately 80 kD. Ng-CAM mAbs immunopurify a 135 kD band and diffuse bands around 80 and 65 kD. The 140/130 kD doublet purified by Bravo mAbs (Bravo-140/130) and the higher molecular weight bands of L1 and Ng-CAM have faster mobilities under nonreducing conditions than under reducing conditions (Fig. 4), implying that these molecules are stabilized by internal disulphide bridges. This apparent molecular weight shift has been described for L1 and NILE, the rat L1 equivalent, and suggests that intrachain disulphide bonds, like those characteristic of immunoglobulin domains, are involved in stabilizing the Bravo protein (Prince et al., 1989).

Both Ng-CAM and L1 are found predominantly as heterodimers, an α polypeptide chain of approximately 140 kD noncovalently attached to a smaller β chain of approximately 80 kD (Fig. 4) (Sadoul et al., 1988; Burgoon et al., 1991). The pattern of Bravo bands in Fig. 4 suggests a similar dimer structure. Anti-Bravo mAbs immunopurify both the 140/130 kD and the 60-80 kD Bravo polypeptides, but only the higher molecular weight bands react with Bravo mAbs on immunoblots, arguing strongly for the heterodimer structure of Bravo (Fig. 5, A and B). This suggests that the immunopurification of the 60-80 kD polypeptides is the result of their strong association with the 130/140 kD polypeptides, as is the case for L1 and Ng-CAM. In the case of L1, the 140 and 80 kD components of the major heterodimer structure represent products of proteolytic processing of an intact polypeptide chain with an apparent molecular weight of 200 kD. Similarly, Ng-CAM is

synthesized as a 200 kD chain, and subsequently processed into the 135 kD α chain and the 80 kD β chain components. L1 (Sadoul et al., 1988) and Ng-CAM mAbs (Fig. 4) can be used to immunopurify these higher molecular weight intact molecules. In contrast, no higher molecular weight proteins were immunopurified using Bravo mAbs. Nonetheless, a Bravo-reactive band of M_r 190 kD was found by immunoblotting components of the tectum insoluble in our lysis buffer (Fig. 5D). Presumably, this Bravo precursor is more thoroughly or rapidly processed into the α and β chains than is the case for L1 and Ng-CAM.

Immunoblotting of purified Bravo protein also reveals that this molecule carries the HNK-1 carbohydrate epitope found on several other neural molecules as well. The HNK-1 epitope is associated with cell-adhesion molecules of the vertebrate nervous system, and may itself play a role in adhesion (Kunemund et al., 1988; Kadmon et al., 1990b). L1, Ng-CAM, Myelin Associated Glycoprotein (MAG), and Neural Cell Adhesion Molecule (N-CAM) among others, are known to carry this epitope (Cole and Schachner, 1987; Kruse et al., 1984). As shown in Fig. 5C, it is the 130-140 kD components of both Bravo and Ng-CAM--not the 60-80 kD ones--that carry this epitope, analogous to the situation with L1 (Faissner et al., 1985).

Isolation of cDNA Clones

The initial identification and isolation of a Bravo cDNA clone was achieved by screening an unamplified λZapII cDNA library prepared using mRNA from E17 chicken cerebella. Eight anti-Bravo mAbs were pooled and used to select Bravo-1 which was subsequently plaque purified and found to react with four of the eight mAbs.

After excision of the insert and DNA sequencing, Bravo-1 was found to encode an open reading frame with neither a start methionine nor a stop codon. Therefore, we rescreened the cerebellar library with the Bravo-1 insert and isolated several clones. These clones were mapped by PCR using specific Bravo-1 primers in conjunction with vector primers to determine which clones had inserts containing the greatest amount of new sequence

data. N1 and C1 were derived in this way and contained sequences more N-terminal and C-terminal to Bravo-1 respectively. Another rescreen with C1 produced C2 and C3 which contained alternative 3' coding regions (Fig. 6a).

The five overlapping Bravo clones (Bravo-1, N1, C1,C2 and C3) were sequenced, and the amino acid sequences of the corresponding polypeptides were deduced. The deduced amino acid sequence was then compared to the protein sequence data generated by analyses of immunopurified Bravo polypeptides. Microsequencing of the first nineteen residues of each component of the purified Bravo 140/130 doublet yielded identical sequences for the Bravo α chains. Peptide sequencing of two proteolytic fragments generated by V8 protease treatment of purified Bravo α chains yielded thirty-five residues of unambiguous amino acid sequence data, and N-terminal sequencing of the 60-80 kD Bravo β chain yielded thirty-five residues of amino acid sequence information. The sequences of these components were found in the translated sequence of Bravo cDNAs, with two amino acid residue exceptions, which we conclude represent amino acid sequencing errors. These putative sequencing errors excepted, the remaining protein data matched the derived sequence exactly at 87 out of 87 sites (Fig. 7). We conclude that the cDNAs we have identified encode Bravo.

cDNA Sequence Analysis and Comparison to L1 and Ng-CAM

As deduced earlier (de la Rosa et al., 1990; Kayyem, J. F., J. M. Roman, E. J. de la Rosa, U. Schwarz and W. J. Dreyer. 1990. *J. Neurosci. Abstracts* 16:1010), Bravo is a member of the immunoglobulin superfamily with close relationship to L1 and Ng-CAM. Another molecule similar to Ng-CAM was recently characterized and given the name Nr-CAM (Grumet et al.,1991). The cDNA sequence of Nr-CAM contains many regions identical in sequence to Bravo, though several novel regions of alternatively spliced sequences exist as well. The cDNA sequence determined by Grumet et al. can be found in the EMBL/Genbank/DDBJ under accession number X58482. Novel sequences identified in this

laboratory are shown in Fig. 6b. Here, we concentrate on results not shown previously, such as the heterodimer nature of Bravo and the novel regions of putative alternative mRNA splicing.

A comparison of Bravo amino acid sequence to those in the translated Genbank sequence database using the FASTA rapid homology search showed that Bravo is most closely related to L1 (Moos et al., 1988) and to Ng-CAM (Burgoon et al., 1990). It is also related to other neural CAMs. The extracellular portion of Bravo encodes six immunoglobulin-like domains and five FNIIIs. The Ig-like domains resemble those found in immunoglobulins to some extent, but more closely resemble those found in the Ig-CAMs (Edelman et al., 1987; Salzer and Colman, 1989). All the Ig-CAMs, Bravo included, contain multiple Ig domains of category C2 (Williams and Barclay,1988). These domains are approximately 100 amino acids long, making them shorter by some 20 amino acids than the V class characteristic of Ig binding domains.

Bravo is shown aligned with L1 and Ng-CAM in Fig. 7, with identical residues boxed. Within each Ig domain, different regions show higher or lower degrees of conservation among the three molecules. For example, the second and third Ig domains show greatest similarity to each other among all three molecules than do the others; the sixth Ig domain shows the least sequence similarity. The high degree of conservation of the second Ig domain may be significant, since the second Ig domain of the neural cell adhesion molecule N-CAM is thought to be involved in both homophilic adhesion (Cunningham et al., 1987) and heparin binding (Cole and Akeson, 1989).

Bravo cDNAs as well as those encoding Nr-CAM show regions of common sequence interrupted by stretches of sequence unique to one or more clone but absent from others, presumably resulting from alternative processing of mRNA. For example, between the second and third immunoglobulin domains lies a stretch of amino acids identified in one Nr-CAM clone (Grumet et al., 1991) and not found in any Bravo clones. This presumably alternatively spliced sequence encodes 20 amino acids and is given the name Alternative Sequence, encoding 20 amino acids (AS20) (Figs. 7 and 8). In addition,

following the sequence encoding the six Ig domains is a 30 base pair stretch of novel sequence found in Bravo clone C1 and N1 but not in either Bravo-1 or C2. This sequence, highly hydrophobic in profile (Kyte and Doolitle, 1982) encodes ten amino acids (Ala-Pro-Pro-Thr-Pro-Ala-Ile-Ile-Tyr-Ala) and is denoted AS10. Following the AS10 alternatively spliced sequence are five fibronectin type III repeats, similar to the repeats found in fibronectin but far more closely related to those of the neural CAMs. As with the Ig-like domains, the degree of similarity of the FNIIIs to each other varies, with the second repeat (FNIII#2) showing the highest level of similarity among Bravo, Ng-CAM and L1, and the fifth repeat (FNIII#5) showing the lowest. Furthermore, the fifth fibronectin repeat (AS93) (also identified as a putative alternatively spliced sequence by Grumet, et al., 1991) is missing from Bravo clone C2, as is AS12, an additional stretch of novel sequence abutting the N-terminus of AS93 (Figs. 6, 7 and 8).

The deduced Bravo sequence and comparison to that of L1 and Ng-CAM also suggests a common mechanism for the generation of the α and β chains from an intact polypeptide. The third fibronectin repeat of Bravo contains the sequence previously identified as the N-terminus of the 60-80 kD β chain of Bravo. As expected from the similarity of Bravo to L1 and Ng-CAM, immediately N-terminal to this site is a conserved series of amino acids culminating in the sequence Ser-Lys-Arg in both L1 and Bravo, and Ser-Arg-Arg in Ng-CAM, suggesting cleavage at this site in all three molecules by a trypsin-like protease. Thus, not only is the heterodimer structure of Bravo similar to that of L1 and Ng-CAM, but so is the mechanism that generates the α and β chains: proteolytic cleavage of an intact precursor molecule at the C-terminal end of the consensus sequence Ser-Arg/Lys-Arg. Bravo contains this putative consensus sequence tandemly repeated at this conserved cleavage site (i.e., Ser-Arg-Arg-Ser-Lys-Arg), which may explain the absence of detectable intact Bravo 190 kD precursor in normal tissue lysates.

The deduced Bravo sequence reveals numerous potential sites of N-linked glycosylation (Asn-X-Ser/Thr) spread throughout the extracellular portion of Bravo. These sites number eighteen within

the Bravo sequence proper, and two within the putative alternatively spliced region AS20. The deduced Bravo sequence also contains a transmembrane domain and a relatively short cytoplasmic domain of 133 amino acids, much of which is highly conserved with Ng-CAM and L1 but which has no known structural motif. Because L1 is phosphorylated *in vivo,* and thought to co-purify with at least one kinase (Faissner et al., 1985; Sadoul et al., 1989; Schuch et al., 1989), the primary sequence of Bravo was analyzed for potential kinase substrates (Glass et al., 1986; Kishimoto et al., 1985; Kuenzel et al., 1987). Eight potential phosphorylation sites are present within the cytoplasmic region where L1 has been shown to be phosphorylated. Three of Bravo's eight potential cytoplasmic phosphorylation sites may be conserved: two align with sites on L1 (Bravo serines 1225 and 1248) and an additional one matches with sites on both L1 and Ng-CAM (Bravo threonine 1173).

The putative alternatively spliced cytoplasmic sequences, AS-CYT1 and AS-CYT2, contain no consensus sequence for known kinases. AS-CYT1 produces a shift of frame that results in a stop codon within the cytoplasmic region of Bravo, and AS-CYT2, 12 base pairs in length, inserts the amino acid sequence Arg-Ser-Leu-Glu (RSLE, using the one letter amino acid code) into Bravo's cytoplasmic domain. This tetrapeptide sequence aligns with the identical sequence in the derived protein sequence of Ng-CAM and is found alternatively spliced in both rat and human L1 (Miura et al., 1991; Kobayashi et al., 1991).

Discussion

We have characterized the Bravo glycoprotein and found that it is related in structure and primary sequence to the cell-surface adhesion molecules Ng-CAM in chicken and L1 in mouse. While the primary structure of Bravo has been previously described under the name Nr-CAM, the structure shown below differs from the structure previously predicted (Grumet *et al.*, 1991), specifically with regard to the apparent molecular weight of the Bravo protein and its heterodimer nature. Bravo is composed of two different chains. The α chain is found as a doublet of 140/130 kD, containing the six Ig-like domains and two complete and one partial FNIII. The Bravo β chain contains two complete and one partial FNIII as well as the transmembrane spanning domain and C-terminal cytoplasmic sequences. These two chains are found associated by noncovalent interactions, and are generated from an intact precursor molecule by proteolytic cleavage following the sequence Ser-Lys-Arg within FNIII#3.

In addition to conserved structural features, the molecular weight of Bravo matches that of L1 and Ng-CAM. Bravo's apparent molecular weight as determined by SDS PAGE of the α and β chains ranges from 190 kD (α chain of 130 kD plus β chain of 60 kD) to 220 kD (α chain of 140 kD plus β chain of 80 kD). Additional evidence for this molecular weight comes from the identification of a putative Bravo precursor molecule detected at 190 kD by immunoblotting the more insoluble components of the tectal membrane. These apparent molecular weight determinations are significantly higher than the predicted molecular weights of polypeptides encoded by the cDNA sequences. Ignoring the molecular weight heterogeneity generated by the putative alternatively spliced sequences AS-CYT1 and AS-CYT2, the predicted molecular weight for Bravo lacking any alternatively spliced sequences is 127 kD. The Bravo protein containing all four of the extracellular alternatively spliced sequences identified has a predicted molecular weight of 142 kD. The difference in these molecular weights from the apparent protein values is presumably due largely to glycosylation. Consistent with

this proposal, Bravo carries the HNK-1 carbohydrate epitope, and its sequence contains twenty potential sites for N-linked glycosylation. Similarly, L1 has a predicted polypeptide molecular weight of only 139 kD and an observed M_r of 200 kD for the intact chain; likewise, Ng-CAM cDNA encodes a polypeptide of 139 kD with an observed M_r of 210/190 kD. The apparent determination of the Nr-CAM molecular weight on SDS PAGE as only 145 kD (Grumet *et al.*, 1991) results from the absence of the 60-80 kD β chain in their protein analyses, and the subsequent failure to account for the substantial increase in apparent molecular weight of the polypeptide caused by glycosylation.

The cleavage of L1, Ng-CAM and Bravo at identical locations (within FNIII#3) and at conserved sequences (Ser-Arg/Lys-Arg) may suggest these molecules are substrates for a common protease. Serine proteases and their inhibitors have previously been implicated in affecting cell migration and neurite outgrowth. Thrombin, which generally cleaves after arginine residues, causes neurites to retract in cultured neuroblastoma cells, while several other proteases do not (Gurwitz and Cunningham, 1988). It has recently been shown that neurons and possibly glia express prothrombin transcripts (Dihanich et al., 1991), and inhibitors of several proteases have been identified both on the surface of and secreted from glioblastoma cells (Wagner et al., 1991). This suggests that regulated proteolysis may be yet another means by which development of the nervous system is facilitated. Moreover, the presence in the Golgi apparatus of proteases that cleave specifically after two basic amino acids (as is seen with L1, Ng-CAM and Bravo) has been implied by investigations of the processing of pro-albumin and parathyroid pro-hormone and several viral structural proteins as well (Rice and Strauss, 1981). Indeed, the cleavage sites of Bravo and Ng-CAM exactly match proteolytic cleavage sites found in alphavirus and flavivirus glycoproteins respectively (Strauss et al., 1987). The cleavage at such a dibasic site within a FNIII may be a common mechanism to produce noncovalently associated heterodimers. The insulin receptor, a mosaic cell-surface protein that includes multiple FNIIIs, is found

as a heterodimer generated from an intact polypeptide by cleavage following the sequence: Ser-Arg-Lys-Arg-Arg, remarkably similar to Bravo's Ser-Arg-Arg-Ser-Lys-Arg (Ebina et al., 1985). While the function of cleavage at this site and the possibly different roles of intact versus heterodimer structures in these molecules has not yet been elucidated, it appears in these cases that this dibasic site is used for processing of a pre-protein form.

The similar structures of Bravo, Ng-CAM and L1, including the proteolytic cleavage site, creates a subgroup of neural Ig-superfamily members. Pairwise sequence comparisons support such a grouping, as these three molecules (and their equivalents) are more closely related to each other than to any as yet reported molecules. The results of a pairwise comparison of these and other vertebrate neural molecules are shown graphically represented as a "gene tree" (Fig. 9). Quantitative sequence identity comparisons (shown by the left to right position of "branch" points) supported by the arrangement of conserved domains among these molecules suggest the division of neural Ig-superfamily members into three subgroups. N-CAM represents the first subgroup; it is characterized by five Ig-like domains and two fibronectin type III repeats. Chicken N-CAM and mouse N-CAM are believed to be equivalent molecules because, in part, they have undergone only 19% divergence (they are 81% identical) since the separation of mammals and birds (Cunningham et al., 1987; Santoni et al., 1987). The next subgroup consists of two pairs of molecules. F3 and Contactin/F11 are thought to be equivalent molecules in mouse and chicken, respectively (Gennarini et al., 1989; Ranscht et al., 1988; Brümmendorf et al., 1989). Likewise, rat TAG-I and chicken axonin-1, are also thought to be equivalent, although the complete characterization of the axonin-1 cDNA has not yet been reported (Furley et al., 1990). With each of these pairs, the equivalent molecules are roughly 80% identical to each other. The layout of these molecules is six Ig domains, four FNIIIs and a glycosylphosphatidylinositol linkage to the membrane.

In contrast, the pairwise comparisons of L1, Ng-CAM and Bravo do not reveal any equivalency among these molecules. The gene tree

in Fig. 9 represents clearly that neither Ng-CAM nor Bravo is equivalent to L1 as the "tri-furcation" of these three molecules is displaced far to theleft (lower percent identity) of the bifurcation of the other molecules. Mouse L1 is equally related at the level of sequence identity (40%) to both chicken Ng-CAM and Bravo. The application of molecular clocks to related molecules in setting up phylogenetic trees relies on the premise that *equivalent* molecules diverge at a relatively constant rate over a given length of time in a particular group of organisms (Dayhoff et al., 1969). The roughly 20% divergence of N-CAM, TAG-1 and F3 from their chicken equivalents over the same amount of time conforms to this premise. The much larger disparity (60%) of L1 and Ng-CAM and of L1 and Bravo suggests that neither Ng-CAM nor Bravo is the L1 equivalent.

Indeed, though many authors had previously viewed Ng-CAM and L1 as equivalent molecules in different species, some results had showed that their roles might be distinct. Ng-CAM, for example, was convincingly shown to be involved in neuron-neuron binding via a homophilic mechanism, and neuron-glia binding via a heterophilic partner (Grumet and Edelman, 1984, 1988; Grumet et al., 1984a,1984b). L1, however, has been shown to be absent from sites of neuron-glia contact and is considered not to be involved in granule cell migration on glia (Persohn and Schachner, 1987). These apparent differences between L1 and Ng-CAM and possibly others (for example the implied interaction of L1 and not Ng-CAM with N-CAM) (Kadmon et al., 1990a) have been the source of some debate. Such differences and the low level of sequence identity between Ng-CAM and L1 support our thesis that these molecules are not equivalent. Thus differences in the functions of these three molecules should be expected.

While the non-equivalence of these molecules seems clear, the exact evolutionary relationship among them is not. The large percent non-identity suggests the possibility that these molecules represent three distinct family members that diverged long before the separation of birds and mammals. Such an ancient origin for the different molecules implies that both chickens and mice might carry a descendent of each of the three ancestral molecules. That is,

there would exist a chicken equivalent of mouse L1 (with approximately 80% sequence identity to mouse L1), and comparable mouse equivalents of both Ng-CAM and Bravo. That these molecules have not been found does not discredit this hypothesis, as the search for such molecules has not been exhaustive. Furthermore, any missing molecule could have been deleted over evolutionary time in a particular species. Another hypothesis not requiring us to posit undiscovered (or extinct) molecules would hold that a common ancestor of Ng-CAM and Bravo duplicated and diverged in a chicken ancestor after birds and mammals diverged. After this hypothetical duplication and some mutation, the chicken ancestor would contain two closely related molecules, and the mouse ancestor only one, so no strict equivalence would exist. Thus, Bravo and Ng-CAM would be expected to diverge at a rate not predictable by rates of equivalent molecules, such as F11/Contactin and F3. The high level of divergence seen among L1, Ng-CAM and Bravo would therefore reflect not an ancient separation of these molecules, but rather a recent (since the divergence of birds and mammals) and rapid accumulation of amino acid substitutions in Ng-CAM and Bravo. The more general paradigm for gene duplication and divergence predicts that one molecule remains equivalent (redundant) to L1 and the other diverges rapidly. Even so, it is not unreasonable to assume that in this case both molecules (Bravo and Ng-CAM) might diverge rapidly from L1 because of the multiple domain nature of these molecules which allows mutations to be accumulated in one domain in one molecule without affecting other, still functional and redundant domains. The discovery of new equivalent molecules in chicken or mouse, and a more general phylogenetic survey of other vertebrates and invertebrates, should help resolve this history.

The close relationship of Bravo, Ng-CAM and L1, especially at the level of higher order protein structure, suggests their roles may be similar. L1 and Ng-CAM have both been implicated in neuron-neuron adhesion, axon fasciculation and neurite outgrowth (Chang et al., 1987; Fischer et al., 1986; Grumet and Edelman, 1988; Grumet et al., 1984a; Lagenaur and Lemmon, 1987; Lemmon et al., 1989; Stallcup and Beasley, 1985), and the drosophila molecule neuroglian,

of a similar structure to the L1 subgroup of Ig-CAMs discussed here, facilitates cell-cell adhesion when transfected into non-adhesive cell lines (Hortsch and Bieber, 1991). Therefore, when combined with the immunolocalization of Bravo, it seems likely that Bravo plays a role in neural adhesion and fasciculation.

The possibly extensive use of alternative splicing may suggest a complex developmentally regulated role for Bravo. In analyzing only five clones in this laboratory, five putative alternatively spliced sequences were identified, not counting AS20 identified previously (Grumet et al., 1991). Alternative splicing of mRNA precursors is generally recognized as providing an important mechanism for generating developmentally programmed functional diversity of proteins involved in cell-surface recognition (e.g., Smith et al., 1989). The specific sequences that may be included or excluded in a particular isoform have, in several examples, been characterized as specific functional protein domains that contribute to different cell adhesion properties. It is interesting to note that in many cases studied so far, a frequent observation is that exons coding for FNIIIs in particular, are expressed in spatiotemporally restricted patterns. Examples include tenascin, fibronectin, and collagenase (Huhtala et al., 1990; Komoriya et al., 1991; Siri et al., 1991; Weller et al., 1991). The programmed alternative splicing of FNIIIs and numerous other functional protein domains is a powerful mechanism for generating functional diversity among cell-surface receptors during development (Kimizuka et al., 1991). The alternatively spliced FNIII repeat from fibronectin, for example, contains two peptide recognition sequences active in cell adhesion: Arg-Glu-Asp-Val and Leu-Asp-Val which bind the $\alpha_4\beta_1$ integrin receptor, rather than the $\alpha_5\beta_1$ integrin receptor of the fibronectin central cell-binding sequence Arg-Gly-Asp (reviewed in D'Souza et al., 1991). It is therefore interesting to note that although Bravo contains no known integrin peptide recognition sequences within its FNIIIs (Yamada, 1991), the entire fifth FNIII is encoded by an alternatively spliced exon, AS93, and can therefore be developmentally modulated.

In Bravo, and in N-CAM (reviewed in Edelman and Crossin,

1991), a number of alternative splicing events appear to affect shorter stretches of amino acids between known larger functional domains. Within the cDNA sequence coding for the Bravo α chain are two such regions, AS20 and AS10. Bravo mRNAs either containing or missing these sequences may correspond to the two forms of the Bravo α chain detected, the 140kD and 130 kD forms. While the 30 amino acids of AS20 combined with AS10 would be expected to produce a molecular weight shift of only 3 kD, the possible glycosylation of two asparagines within AS20 (which produces profoundly greater molecular weight shifts than similarly sized protein side chains) suggests a source for the observed 10 kD difference in apparent molecular weight. AS10, flanked by the six Ig-like domains on one side and the five FNIIIs on the other, lies in a region analogous to the hinge region of the neural cell adhesion molecule N-CAM (Becker et al., 1989). This region has been implicated in cis-interactions of N-CAM with both itself and other membrane molecules such as L1. In addition, antibodies against this region disrupt optic fiber fascicles and cause misrouting of fibers in the retina (Pollerberg and Dreyer, submitted).

Bravo also contains alternatively spliced sequences within the highly conserved cytoplasmic domain. The determinations of Bravo's genomic DNA sequence and further peptide sequence analyses will be necessary, however, to determine whether these and the other alternative cDNA sequences represent functional Bravo variants rather than misprocessed mRNA. The sequence of AS-CYT1 in particular suggests the need for further analysis, as it encodes a frameshift resulting in a truncated derived protein sequence. In addition, AS-CYT1 and AS-CYT2 both show similarity to 3' regions of introns, including potential polypyrimidine tracks and termination with the sequence AG (Smith et al., 1989). On the other hand, in support of the notion that AS-CYT2 is not an artifact of cloning but actually encodes a functional Bravo variant, it is interesting to note that both human and rat versions of L1 contain alternatively spliced sequences corresponding exactly to the tetrapeptide encoded by AS-CYT2, Arg-Ser-Leu-Glu. Furthermore, L1 from rat shows a tissue-specific regulation of this alternative cytoplasmic sequence: the

isoform missing this sequence is predominantly expressed in the brain, while the RSLE isoform is found in the peripheral nervous system (Miura et al., 1991). Investigations of the distribution of Bravo outside the retinotectal system--specifically of the isoforms containing or missing AS-CYT2--might reveal a similar spatial restriction and suggest some role for alternative splicing in this region, possibly involving alterations of interactions with cytoplasmic or cytoskeletal proteins critical for influencing neurite outgrowth and cell migration.

The close relationship of Bravo to neural CAMs and the restricted pattern of Bravo expression suggests a testable model for Bravo function: Bravo is turned on as retinal fibers are born, facilitating (via interactions with other Bravo molecules and possibly other CAMs such as Ng-CAM) the dense fasciculation and strong neighbor association of these axons as they grow out of the retina and towards the tectum. A change in extracellular or cell-surface environment near the chiasm triggers the termination of Bravo expression on optic fibers as they extend toward their targets in the tectum. This is consistent with the environmental modulation of Bravo expression seen *in vitro*, in which retinal fibers grown on retinal basal lamina containing glial end-feet (their natural retinal substrate) display Bravo antigen; but those grown on alternative substrates such as collagen do not (de la Rosa et al., 1990). In the absence of Bravo protein synthesis, newly forming axon membrane would be initially void of Bravo antigen, and the Bravo protein already displayed on the axon surface might be laterally restricted to older retinal regions of the fiber by extensive interactions with surrounding ECM and cell-surface components (Zhang et al., 1991). This notion is consistent not only with the observed restriction of Bravo along the retinal axon, but also with the observation that Bravo staining of older optic fibers in the retina is lost after PFA fixation, presumably due to masking of the epitopes by covalent crosslinking of proteins surrounding the Bravo antigen.

Under this model, distal and growing parts of the axon would not display new Bravo protein and hence would be reduced in their cohesiveness with other fibers, allowing less hindered target

seeking by the growth cone. In addition, Bravo protein found on glial end-feet in the retina (and possibly in the tectum) might interact with retinal axons via Bravo-Bravo homophilic binding, similar to the predicted interaction facilitated by fasciclin II of glia and axons in insects (Harrelson and Geedman, 1988). The close relationship of Bravo and Ng-CAM suggests that they might interact heterophilically (as do L1 and Ng-CAM with the same 40% sequence identity)(Lemmon et al, 1989). This is consistent with the observation that Ng-CAM mediates neuron-glia adhesion via a heretofore unidentified glial protein (Grumet et al., 1984a). Given the results described here, it will be possible to test the role of Bravo and its different isoforms in neuron-neuron and neuron-glia adhesion by means of several novel assays (*e.g.*, Hortsch and Bieber, 1991; Lee and Lander, 1991). The large number of domains and alternatively spliced sequences--both extra- and intracellularly--suggests numerous possible functions of Bravo.

References

Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB J.* 4:2868-2880.

Ansorge, W. 1985. Fast and sensitive detection of protein and DNA bands by treatment with potassium permanganate. *J. Biochem. Biophys. Methods.* 11:13-20.

Becker, J. W., H. P. Erickson, S. Hoffman, B. A. Cunningham, and G. M. Edelman. 1989. Topology of cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 86:1088-1092.

Bieber, A. J., P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. 1989. Drosophila Neuroglian: A member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell* 59:447-460.

Bork, P. 1991. Shuffled domains in extracellular proteins. *FEBS Letters.* 286:47-54.

Brümmendorf, T., J. M. Wolff, R. Frank, and F. G. Rathjen. 1989. Neural cell recognition molecule F11: Homology with fibronectin type III and immunoglobulin type C domains. *Neuron* 2:1351-1361.

Burgoon, M. P., M. Grumet, V. Mauro, G. M. Edelman, and B. A. Cunningham. 1991. Structure of the chicken neuron-glia cell adhesion molecule, Ng-CAM: Origin of the polypeptides and relation to the Ig superfamily. *J. Cell Biol.* 112:1017-29.

Carter, C., V. J. Britton, and L. Haff. 1983. CsTFA: A centrifugation medium for nucleic acid isolation and purification. *BioTechniques* Sept/Oct:142-147.

Chiquet-Ehrismann, R. 1991. Anti-adhesive molecules of the

extracellular matrix. *Curr. Op. Cell Biol.* 3:800-804.

Chang, S., F. G. Rathjen, and J. A. Raper. 1987. Extension of neurites on axons is impaired by antibodies against specific neural cell surface glycoproteins. *J. Cell Biol.* 104:355-362.

Chuong, C. M., and G. M. Edelman. 1985. Expression of cell-adhesion molecules in embryonic induction II: Morphogenesis of adult feathers. *J Cell Biol.* 101:1027-43.

Cole, G. J., and R. Akeson. 1989. Identification of a heparin binding domain of the neural cell adhesion molecule N-CAM using synthetic peptides. *Neuron* 2:1157-1165.

Cole, G. J., and M. Schachner. 1987. Localization of the L2 monoclonal antibody binding site on chicken neural cell adhesion molecule (NCAM) and evidence for its role in NCAM-mediated cell adhesion. *Neurosci. Lett.* 78:227-232.

Crossin, K. L., C. -M Chuong, and G. M. Edelman. 1985. Expression sequences of cell adhesion molecules. *Proc. Natl. Acad Sci. USA* 82:6942-6946.

Crossland, W. J., W. M. Cowan, and L. A. Rogers. 1975. Studies on the development of the chick optic tectum, IV: An autoradiographic study of the development of the retinotectal connections. *Brain Res.* 91:1-23.

Cunningham, B. A., J. J. Hemperly, B. A. Murray, E. A. Prediger, R. Brackenbury, and G. M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science (Wash. DC).* 236:799-806.

Dayhoff, M. O., R. M. Schwartz, and B. C. Orcutt. 1969. A model of evolutionary change in proteins. *In* Atlas of Protein Sequence and Structure, Vol. 4. M. O. Dayhoff, ed., Silver Springs, Md.:Natl. Biomed.

Res. Found.

de la Rosa, E. J., J. F. Kayyem, J. M. Roman, Y. D. Stierhof, W. J. Dreyer, and U. Schwarz. 1990. Topologically restricted appearance in the developing chick retinotectal system of Bravo, a neural surface protein: Experimental modulation by environmental cues. *J. Cell Biol.* 111:3087-3096.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids. Res.* 12:387-395.

Dihanich, M., M. Kaser, E. Reinhard, D. Cunningham, and D. Monard. 1991. Prothrombin mRNA is expressed by cells of the nervous system. *Neuron* 6:575-581.

Dodd, J., S. B. Morton, D. Karagogeos, M. Yamamoto, and T. M. Jessell. 1988. Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron.* 1:105-116.

Doolittle, R. F. 1985. The geneology of some recently evolved vertebrate proteins. *Trends Biochem. Sci.* 10:233-237.

D'Souza, S. E., M. H. Ginsberg, and E. F. Plow. 1991. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. *Trends Biochem. Sci.* 16:246-250.

Ebina, Y., L. Ellis, K. Jarnagin, M. Edery, L. Graf, E. Clauser, J.-H. Ou, F. Masiarz, Y. W. Kan, I. D. Goldfine, R. A. Roth, and W. J. Rutter. 1985. The human insulin receptor cDNA: The structural basis for hormone-activated transmembrane signalling. *Cell.* 40L747-758.

Edelman, G. M. 1986. Cell adhesion molecules in neural histogenesis. *Ann. Rev. Physiol.* 48:417-430.

Edelman, G. M. 1987. CAMs and Igs: Cell adhesion and the

evolutionary origins of immunity. *Immunol. Rev.* 100:11-45.

Edelman, G. M., and K. L. Crossin. 1991. Cell adhesion molecules: Implications for a molecular histology. *Annu. Rev. Biochem.* 60:155-190.

Edelman, G. M.and J. P. Thiery. 1990. The Cell in Contact: Adhesions and Junctions as Morphogenic Determinants. John Wiley & Sons, Inc. New York.

Engel, J. 1991. Domains in proteins and proteoglycans of the extracellular matrix with functions in assembly and cellular activities. *Int. J. Biol. Macromol.* 13:147-151.

Faissner, A., D. B. Teplow, D. Kubler, G. Keilhauer, V. Kinzel, and M. Schachner. 1985. Biosynthesis and membrane topography of the neural cell adhesion molecule L1. *EMBO J.* 4:3105-3113.

Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266-267.

Fischer, G., V. Kunemund, and M. Schachner. 1986. Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell surface glycoprotein L1. *J. Neurosci.* 6:605-612.

Furley, A. J., S. B. Morton, D. Manalo, D. Karagogeos, J. Dodd, and T. M. Jessell. 1990. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite-promoting activity. *Cell* 61:157-170.

Gennarini, G., G. Rougon, F. Vitiello, P. Corsi, C. D Benedetta, and C. Goridis. 1989. Identification and cDNA cloning of a new member of the L2/HNK-1 family of neural surface glycoproteins. *J. Neurosci. Res.* 22: 1-12.

Glass, D. B., M. R. El-Maghrabi, and S. J. Pilkis. 1986. Synthetic peptides corresponding to the site phosphorylated in 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase as substrate of cyclic nucleotide-dependent protein kinases. *J. Biol. Chem.* 261:2987-2993.

Grumet, M., and G. M. Edelman. 1984. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J Cell Biol* 98:1746-56.

Grumet, M., and G. M. Edelman. 1988. Neuron-glia cell adhesion molecule interacts with neurons and astroglia via different binding mechanisms. *J. Cell Biol.* 106: 487-503.

Grumet, M., S. Hoffman, C. -M Chuong, and G. M. Edelman. 1984a. Polypeptide components and binding functions of neuron-glia cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* 81:7989-7993.

Grumet, M., S. Hoffman, and G. M. Edelman. 1984b. Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron-neuron and neuron-glia adhesion. *Proc. Natl. Acad. Sci. USA* 81:267-271.

Grumet, M., V. Mauro, M. P. Burgoon, G. E. Edelman, and B. A. Cunningham. 1991. Structure of a new nervous system glycoprotein, Nr-CAM, and its relationship to subgroups of neural cell adhesion molecules. *J. Cell Biol.* 113:1399-1412.

Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene (Amst.).* 25:263-269.

Gurwitz, D., and D. D. Cunningham. 1988. Thrombin modulates and reverses neuroblastoma neurite outgrowth. *Proc. Natl. Acad. Sci. USA* 85:3440-3444.

Harrelson, A. L., and C. S. Goodman. 1988. Growth cone guidance in insects: Fasciclin II is member of the immunoglobulin superfamily. *Science* 242:700-708.

Hortsch, M., and A. J. Bieber. 1991. Sticky molecules in not-so-sticky cells. *Trends Biochem. Sci.* 16:283-287.

Huhtala, P., L. T. Chow, and K. Tryggvason. 1990. Structure of the human type IV collagenase gene. *J. Biol. Chem.* 265:11077-11082.

Hunkapiller, T. and L. Hood. 1989. Diversity of the immunoglobulin gene superfamily. *Advances in Immunology* 44:1-63.

Hynes, R. O. 1987. Integrins, a family of cell surface receptors. *Cell* 48:549-555.

Inuzuka, H., S. Miyatani, and M. Takeichi. 1991. R-Cadherin: A novel Ca^{2+} -dependent cell-cell adhesion molecule expressed in the retina. *Neuron* 7:69-79.

Jessell, T. M. 1988. Adhesion molecules and the hierarchy of neural development. *Neuron* 1:3-13.

Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990a. The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. *J. Cell Biol.* 110:193-208.

Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990b. Functional cooperation between the neural adhesion molecules L1 and N-CAM is carbohydrate dependent. *J. Cell Biol.* 110:209-18.

Kimizuka, F., Y. Ohdate, Y. Kawase, T. Shimojo, Y. Taguchi, K. Hashino, S. Goto, H. Hashi, I. Kato, K. Sekiguchi, and K. Titani. 1991. Role of type-III homology repeats in cell adhesive function within the cell-binding domain of fibronectin. *J. Biol. Chem.* 266:3045-3051.

Kishimoto, A., K. Nishiyama, H. Nakanishi, Y. Uratsuji, H. Nomura, Y. Takeyama, and Y. Nishizuka. 1985. Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3',5'-monophosphate dependent protein kinase. *J. Biol. Chem.* 260:1156-1164.

Kobayashi, M., M. Miura, H. Asou, and K. Uyemura. 1991. Molecular cloning of cell adhesion molecule L1 from human nervous tissue: A comparison of the primary sequences of L1 molecules of different origin. *Biochim. Biophys. Acta.* 1090:238-240

Komoriya, A., L. J. Green, M. Mervic, S. S. Yamada, K. M. Yamada, and M. J. Humphries. 1991. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J. Biol. Chem.* 266:15075-15079.

Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature* 311:153-155.

Kuenzel, W. A., J. A. Mulligan, J. Sommercorn, and E. G. Krebs. 1987. Substrate specificity determinants for Casein Kinase II as deduced from studies with synthetic peptides. *J. Biol. Chem.* 262:9136-9140.

Kunemund, V., F. B. Jungalwala, G. Fischer, D. K. H. Chou, G. Keilhauer, and M. Schachner. 1988. The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol.* 106: 213-223.

Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.

Lagenaur, C., and V. Lemmon. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. USA.* 84:7753-7747.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.

Lee, M. K., and A. D. Lander. 1991. Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: Development of a sensitive electrophoretic approach. *Proc. Natl. Acad. Sci. USA.* 88:2768-2772.

Lemmon, V., K. L. Farr, and C. Lagenaur. 1989. L1-mediated axon outgrowth occurs via a homophilic binding mechanism. *Neuron* 2:1597-1603.

Lemmon, V., and S. C. McLoon. 1986. The appearance of an L1-like molecule in the chick primary visual pathway. *J. Neurosci.* 6:2987-2994.

Magee, A. I., and R. S. Buxton. 1991. Transmembrane molecular assemblies regulated by the greater cadherin family. *Curr. Op. Cell Biol.* 3:854-861.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, Cold Spring Harbor, New York.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidine difluoride membranes. *J. Biol. Chem.* 262: 10035-10038.

Miura. M., M. Kobayashi, H. Asou, and K. Uyemura. 1991. Molecular cloning of cDNA encoding the rat neural cell adhesion molecule L1. *FEBS Letters* 289:91-95.

Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Fruh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* 334:701-703.

Pearson, W. R., and D. J. Lipman. 1988. Improved tool for biological sequence comparison. *Proc. Natl. Acad. Sci.* USA 85:2444-2448.

Persohn, E., and M. Schachner. 1987. Immunoelectron microscopic localization of the neural cell adhesion molecules L1 and N-CAM during postnatal development of the mouse cerebellum. *J. Cell Biol.* 105:569-76.

Pesheva, P., E. Spiess, and M. Schachner. 1989. J1-160 and J1-180 are oligodendrocyte-secreted nonpermissive substrates for cell adhesion. *J. Cell Biol.* 109:1765-1778.

Pollerberg, G. E., K. Burridge, K. Krebs, S. Goodman, and M. Schachner. 1987. The 180 kD component of the neural cell adhesion molecule N-CAM is involved in cell-cell contacts and cytoskeleton-membrane interactions. *Cell Tissue Res.* 250:227-236.

Pollerberg, G. E., and W. J. Dreyer. 1992. Cell-cell interactions underlying axonal fasciculation and orientation involve the hinge region of neural cell adhesion molecule (N-CAM). *J. Cell Biol.* submitted for publication.

Prieto, A. L., F. S. Jones, B. A. Cunningham, K. L. Crossin, and G. M. Edelman. 1990. Localization during development of alternatively spliced forms of cytotactin mRNA by *in situ* hybridization. *J. Cell Biol.* 111:685-698.

Prince, J. T., N. Milona, and W. B. Stallcup. 1989. Characterization of a partial cDNA clone for the NILE glycoprotein and identification of the encoded polypeptide domain. *J. Neurosci.* 9:876-883.

Ranscht, B. 1988. Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. *J. Cell Biol.* 107:1561-1573.

Rathjen, F. G., J. M. Wolff, R. Frank, F. Bonhoeffer, and U. Rutishauser. 1987. Membrane glycoproteins involved in neurite fasciculation. *J. Cell Biol.* 104:343-53.

Reichardt, L. F., and K. J. Tomaselli. 1991. Extracellular matrix molecules and their receptors: Functions in neural development. *Annu. Rev. Neurosci.* 14:531-570.

Rice, C. M. and J. H. Strauss. 1981. Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. USA.* 78:2062-2066.

Ruegg, M. A., W. T. Stoeckli, R. B. Lanz, P. Streit, and P. Sonderegger. 1989. A homologue of the axonally secreted protein axonin-1 is an integral membrane protein of nerve fiber tracts involved in neurite fasciculation. *J. Cell Biol.* 109:2363-2378.

Sadoul, K., R. Sadoul, A. Faissner, and M. Schachner. 1988. Biochemical characterization of different molecular forms of the neural cell adhesion molecule L1. *J. Neurochem.* 50:510-521.

Sadoul, R., F. Kirchhoff, and M. Schachner. 1989. A protein kinase activity is associated with and specifically phosphorylates the neural cell adhesion molecule L1. *J. Neurochem.* 53:1471-1478.

Salzer, J. L., and D. R. Colman. 1989. Mechanisms of cell adhesion in the nervous system: role of the immunoglobulin gene superfamily. Dev. Neurosci. 11:377-390.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-

5467.

Santoni, M.-J., D. Barthels, J. A. Barbas, R. Hirsh, C. Goridis, and W. Wille. 1987. Analysis of cDNA clones that code for the transmembrane forms of the mouse neural cell adhesion molecule (NCAM) and are generated by alternative RNA splicing. *Nucleic Acids Res.* 15:8621-8641.

Schuch, U., M. J. Lohse, and M. Schachner. 1989. Neural cell adhesion molecules influence second messenger systems. *Neuron* 3:13-20.

Sherr, C. J., C. W. Rettenmier, R. Sacca, M. F. Roussel, A. T. Look, and E. R. Stanley. 1985 The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 41:665-676.

Siri, A., B. Carnemolla, M. Saginati, A. Leprini, G. Casari, F. Baralle, and L. Zardi. 1991. Human tenascin: primary structure, pre-mRNA splicing patterns and localization of the epitopes recognized by two monoclonal antibodies. *Nucl. Acids Res.* 19:525-531.

Smith, C. W. J., E. B. Porro, J. G. Patton, and B. Nadal-Girard. Scanning from an independently specified branch point defines the 3' splice site of mammalian introns. *Nature* 343:243-247.

Stallcup, W. B., and L. L. Beasley. 1985. Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. *Proc. Natl. Acad. Sci. USA* 82:1276-1280.

Strathmann, M., B. A. Hamilton, C. A. Mayeda, M. I. Simon, E. M. Meyerowitz, and M. J. Palazzolo. 1991. Transposon-facilitated DNA sequencing. *Proc. Natl. Acad. Sci. USA* 88:1247-1250.

Strauss, J. H., E. G. Strauss, C. S. Hahn, Y. S. Hahn, R. Galler, W. R. Hardy, and C. M. Rice. 1987. Replication of alphaviruses and

flaviviruses: Proteolytic processing of polyproteins. *In* Positive Strand RNA Viruses. M. A. Brinton and R. R. Rueckert, editors. Alan R. Liss, New York. 209-225.

Takeichi, M. 1990. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* 59:237-252.

Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science.* 251:1451-1455.

Towbin, H., T. Staehelin, and T. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

Updyke, T. V., and G. L. Nicolson. 1984. Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices. *J. Immunol. Methods.* 73:83-95.

Wagner, S. L., A. L. Lau, A. Nguyen, J. Mimuro, D. J. Loskutoff, P. J. Isackson, and D. D. Cunningham. 1991. Inhibitors of urokinase and thrombin in cultured neural cells. *J. Neurochem.* 56:234-242.

Weller, A., S. Beck, and P. Ekblom. 1991. Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. *J. Cell. Biol.* 112:355-362.

Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily: Domains for cell surface recognition. *Ann. Rev. Immunol.* 6:381-405.

Yamada, K. M. 1991. Adhesive recognition sequences. *J. Biol. Chem.* 266:12809-12812.

Yarden, Y., J. A. Eccobedo, W.-J. Kuang, T. L. Yang-Feng, T. O. Daniel, P. M. Tremble, E. Y. Chen, M. E. Ando, R. N. Harkins, U. Francke, V. A. Fried,

A. Ullrich, and L. T. Williams. 1986. Structure of the receptor for platelet derived growth factor helps define a family of closely related growth factor receptors. *Nature* 323:226-232.

Young, R. A., and R. W. Davis. 1983. Yeast RNA Polymerase III genes: Isolation with antibody probes. *Science (Wash. DC)*. 222:778-782.

Zhang, F., B. Crise, B. Su, Y. Hou, J. K. Rose, A. Bothwell, and K. Jacobson. 1991. Lateral diffusion of membrane-spanning and glycosylphosphatidylinositol-linked proteins: Toward establishing rules governing the lateral mobility of membrane proteins. *J. Cell Biol.* 115:75-84.

Figures and Figure Legends

Figure 1. Distinct immunofluorescence staining patterns of Bravo and Ng-CAM mAbs in the developing chicken retinotectal system. Neighboring sections of E7.5 chicken retina fluorescence were labeled with (a) anti-NgCAM mAb 1E12, or with (b) anti-Bravo mAb, 2B3. Both Ng-CAM and Bravo appear on axons in the optic fiber layer (OFL). In addition, Bravo is distributed throughout the retina, including in the inner plexiform layer (IPL) and newly forming outer plexiform layer (OPL). Note also the apparent Bravo labeling of radial processes in the retina.

Figure 2. Immunofluorescence staining of chicken retina showing labeling of Müller glial end-feet. A region of a cryostat section of an E7.5 retina was found relatively free of optic fiber fascicles. Labeling of this region with anti-Bravo mAb 2B3, and FITC-conjugated anti-mouse Ig reveals intense staining of glial end-feet in the OFL, as well as staining of processes in the IPL and ganglion cell layer (GCL).

Figure 3. Fixation-dependent Bravo labeling of E7.5 retina. Sections of E7.5 retina were prepared as described in Materials and Methods and fixed with either (a) TCA, or (b) PFA. Labeling of these sections with anti-Bravo mAb, 2B3, followed by FITC-conjugated anti-mouse Ig reveals nearly identical staining in the IPL and OPL. A dramatic difference, however, is observed in the OFL where all fibers are labeled evenly after TCA fixation (a), but only surface (vitreal) fibers are labeled after PFA fixation (b).

Figure 4. Ng-CAM and Bravo proteins resolved by SDS PAGE. Immunoaffinity isolates using anti-NgCAM mAbs and anti Bravo mAbs were separated by 7.5% SDS PAGE and visualized by silver staining. Ng-CAM protein was treated to reduce disulphide bonds (lane 1) or treated to

protect these bonds (lane 2); similarly, immunopurified Bravo protein is shown reduced (lane 3) and unreduced (lane 4). Note that the Ng-CAM 135 kD band and the Bravo 140/130 kD doublet appear to contain internal disulphide bonds that cause lower mobility under reducing conditions. A similar effect is seen with the less abundant Ng-CAM 190 kD intact polypeptide, but not with the lower molecular weight Ng-CAM or Bravo 60-80 kD bands.

Figure 5. Comparison of immunoblots of Ng-CAM and Bravo. The immunoaffinity isolates (a, b and c) using anti-NgCAM (lane 1) and anti-Bravo (lane 2) were resolved by 6% SDS PAGE under nonreducing conditions, transferred to nitrocellulose and analyzed by mAb staining with anti-NgCAM (a), anti-Bravo (b) and anti-HNK-1 (c). Proteins solubilized from the tectal lysate pellet (see Materials and Methods) were resolved by 6% SDS PAGE, transferred to modified nitrocellulose membrane, and subjected to mAb analysis with anti-Bravo (d). Binding of all antibodies was visualized using biotinylated second antibodies bound to HRP-conjugated streptavidin. The positions of molecular weight standards (reduced) are shown to the left of each panel.

Figure 6. Schematic representation of Bravo cDNA clones and sequences of novel alternatively spliced regions. (a) The five cDNA clones used to determine the sequence of Bravo are shown. Sequences absent from one or more clones, suggesting alternative processing of messenger RNA, are indicated by angled lines, and labeled AS10, AS12, AS93, AS-CYT1 and AS-CYT2. Bravo-1 was sequenced in its entirety using a transposon based strategy for spreading primer sequences throughout the clone. The remaining clones were sequenced using synthetic oligonucleotide primers in both sense and anti-sense directions. (b) The cDNA (boxed) and derived protein sequences of novel putative alternatively spliced regions AS10, AS12, AS-CYT1 and AS-CYT2 are shown in upper case. Flanking Bravo sequences in lower case are shown for positioning. AS12 and AS-CYT1 were found associated with and abutting alternative sequences AS93 and

AS-CYT2 respectively. Note that these sequences, and AS-CYT2 as well, terminate with the bases AG and contain tracks of polypyrimidines which are associated with intron branch points and 3' intron splice sites, suggesting that these sequences may represent incompletely processed mRNA.

Figure 7. Alignment of the complete amino acid sequences of Bravo, Ng-CAM and L1. The cDNA derived sequence of Bravo is identical to that of Nr-CAM except for the identification of the putative alternatively spliced sequences, AS20 uniquely in Nr-CAM (Grumet et al., 1991) and AS10, AS12, AS-CYT1 and AS-CYT2 uniquely here. AS93 was identified in both laboratories. The Bravo sequence (shown missing AS-CYT1 which encodes a stop codon and therefore results in a truncated Bravo protein) was aligned with L1 and Ng-CAM by eye and gaps introduced where appropriate to maximize identity. Identical residues are boxed. The six immunoglobulin-like domains in all three molecules are labeled Ig I-Ig VI with characteristic cysteines and tryptophans in bold type. The five fibronectin-type III repeats in all three molecules are labeled FNIII#1-FNIII#5 and characteristic tryptophans and tyrosines (or phenylalanines) are also in bold type. The location of the common proteolytic cleavage site is indicated. The N-terminus of the Ng-CAM β chain is removed by one amino acid (Gln, single letter amino acid code Q) from this conserved site. Sequences confirmed by protein and peptide sequencing of Bravo are overlined with two misidentified amino acid residues indicated at positions 2 and 850. A nonstandard amino acid residue labeled "?" was identified at position 861, which is a potential asparagine-linked glycosylation site. The transmembrane domain in all three molecules is underlined with dashed lines. Putative alternatively spliced sequences of Bravo are labeled (e.g., **AS20**) in bold type and bordered by inverted triangles. The sequence found alternatively spliced in rat and human equivalents of L1, and identical to Bravo AS-CYT2, is bordered by triangles.

Figure 8. Topological model of Bravo including alternatively spliced sequences. The six Ig domains of Bravo are shown as loops linked by disulphide (S-S) bonds. The five FNIIIs are shown as darkened boxes. The six potential alternatively spliced regions are shown by hatched boxes labeled AS20, AS10, AS12, AS93, AS-CYT1 and AS-CYT2. Two alternative cytoplasmic domains are shown, one terminating within AS-CYT1 and resulting in a truncated Bravo isoform. Putative asparagine-linked glycosylation sites are indicated with circle-and-stick symbols. The oversized circle-and-stick symbol within FNIII#3 of the Bravo β chain denotes the location of a potential glycosylation site confirmed by amino acid sequence analysis to contain a nonstandard amino acid residue, presumably a glycosylated asparagine. The location of the cleavage site within FNIII#3 is indicated by an arrow. The domains involved in the noncovalent association of the α and β chains are not known.

Figure 9. Gene tree representing subgroups of Ig-CAMs identified by amino acid sequence identity and domain structure. Protein sequences for each of the molecules were compared pairwise using the FASTA program of Pearson and Lipman (1988). A tree of relatedness was constructed with lines branching at positions corresponding to percent sequence identity (plus or minus 1.5%). Models of the three characteristic subgroups are shown to the right. Hypotheses about the relationship among Bravo, Ng-CAM and L1 (shown as a "tri-furcation" of the tree in this figure) are included in the text.

Fig. 1

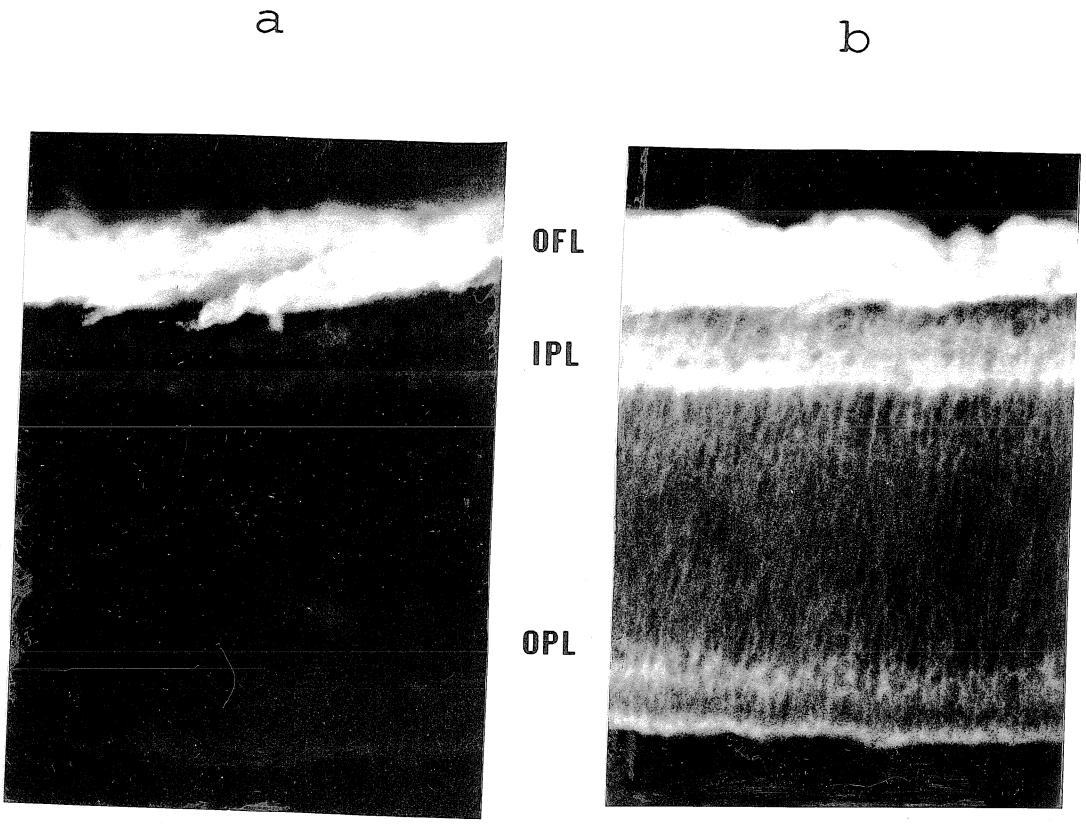


Fig. 2

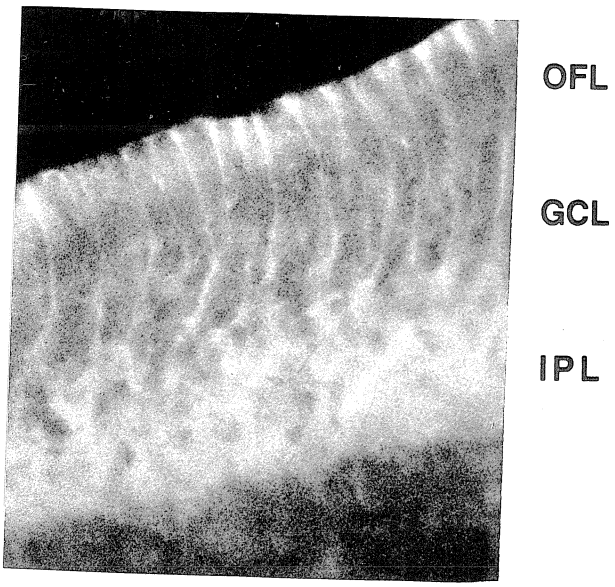
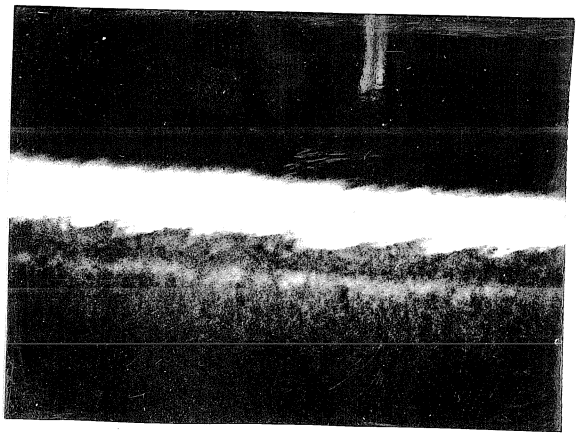


Fig. 3

a



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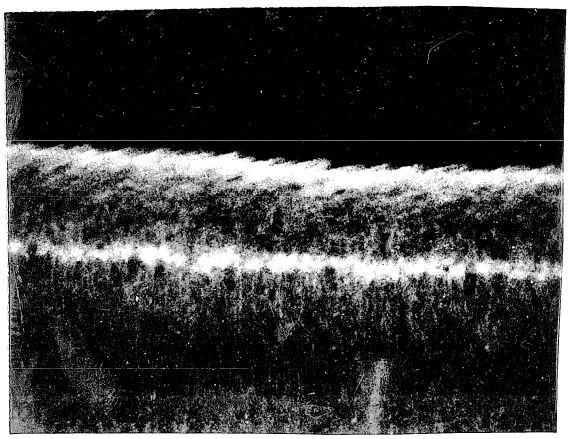


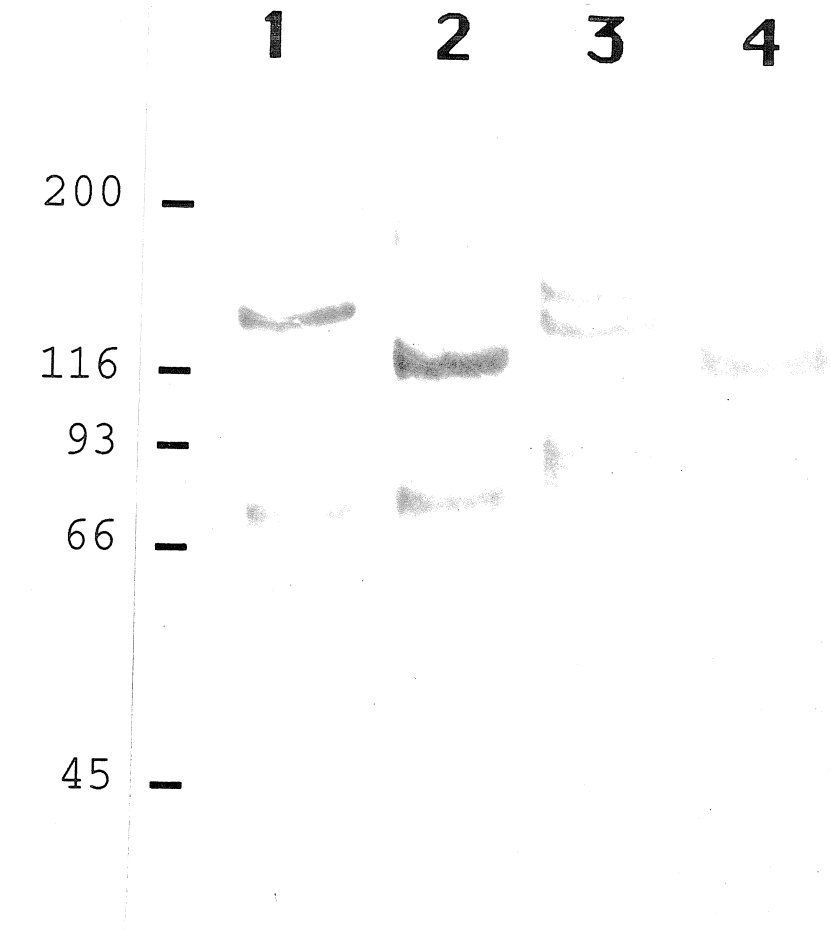
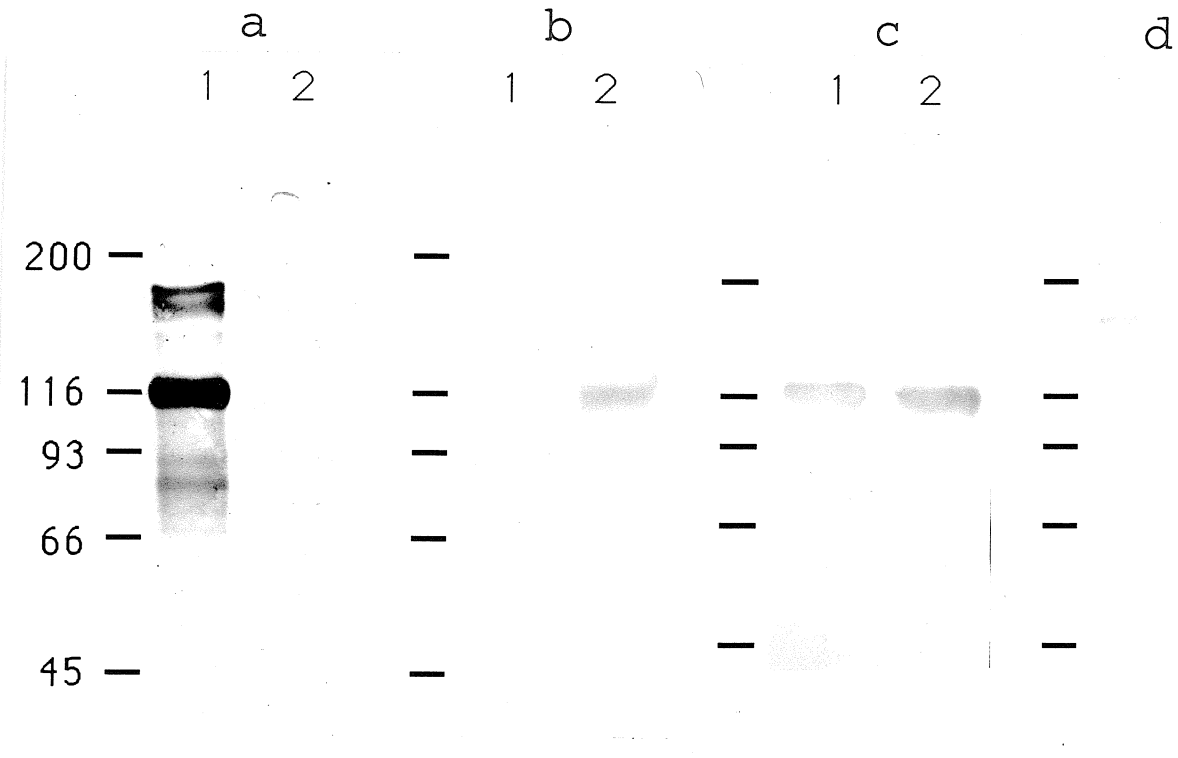


Fig. 4

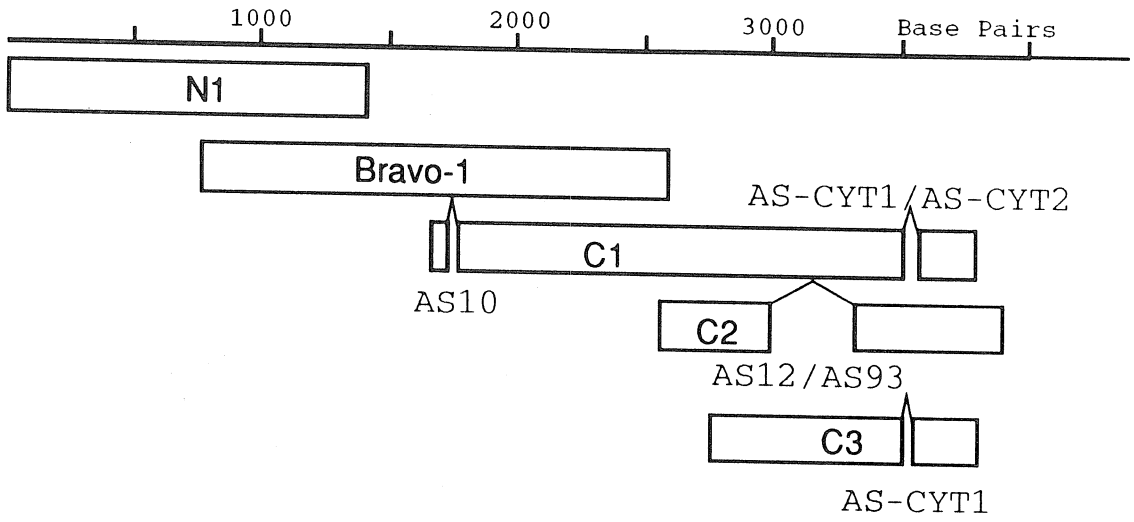
Fig. 5



IV-51

Fig. 6

a



b

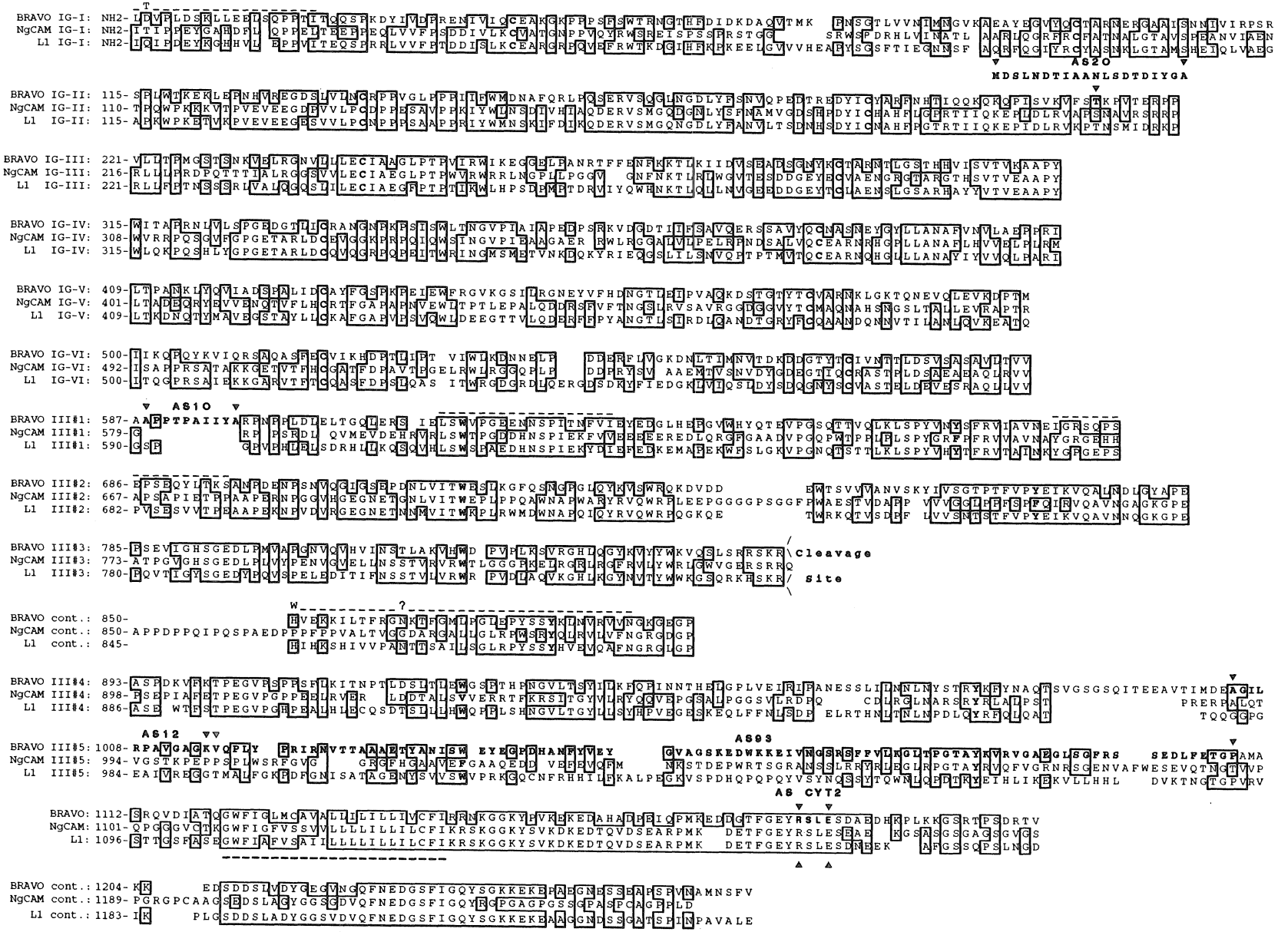
588- a A P P T P A I I Y A r
AS10: 1762- g c t GCTCCCCCAACTCCAGCTATCATTTACGCT c g g

1002- e A G I L R P A V G A G K
AS12: 3004- g a a g CTGGTATTCTTCGTCCTGCTGTAGGTGCAGGCAAAG AS93

1171- y R L F S F V S S A P F Z
AS-CYT1: 3529- t a c a g GCTATTTTCATTTGTTTCTTCTGCACCCTTTTAATGGAATATGTAG AS-CYT2

1171- y R S L E s d
AS-CYT2: 3529- t a c a g GTCTCTTGAAAG t g a t

Fig. 7


BRAVO IG-I:
NgCAM IG-I:
L1 IG-I:
BRAVO IG-II:
NgCAM IG-II:
L1 IG-II:
BRAVO IG-III:
NgCAM IG-III:
L1 IG-III:
BRAVO IG-IV:
NgCAM IG-IV:
L1 IG-IV:
BRAVO IG-V:
NgCAM IG-V:
L1 IG-V:
BRAVO IG-VI:
NgCAM IG-VI:
L1 IG-VI:
AS20
MDSLNDTIAANLSDTDIYGA
AS10
BRAVO III#1:
NgCAM III#1:
L1 III#1:
BRAVO III#2:
NgCAM III#2:
L1 III#2:
BRAVO III#3:
NgCAM III#3:
L1 III#3:
Cleavage
site
BRAVO cont.:
NgCAM cont.:
L1 cont.:
BRAVO III#4:
NgCAM III#4:
L1 III#4:
AS12
AS93
BRAVO III#5:
NgCAM III#5:
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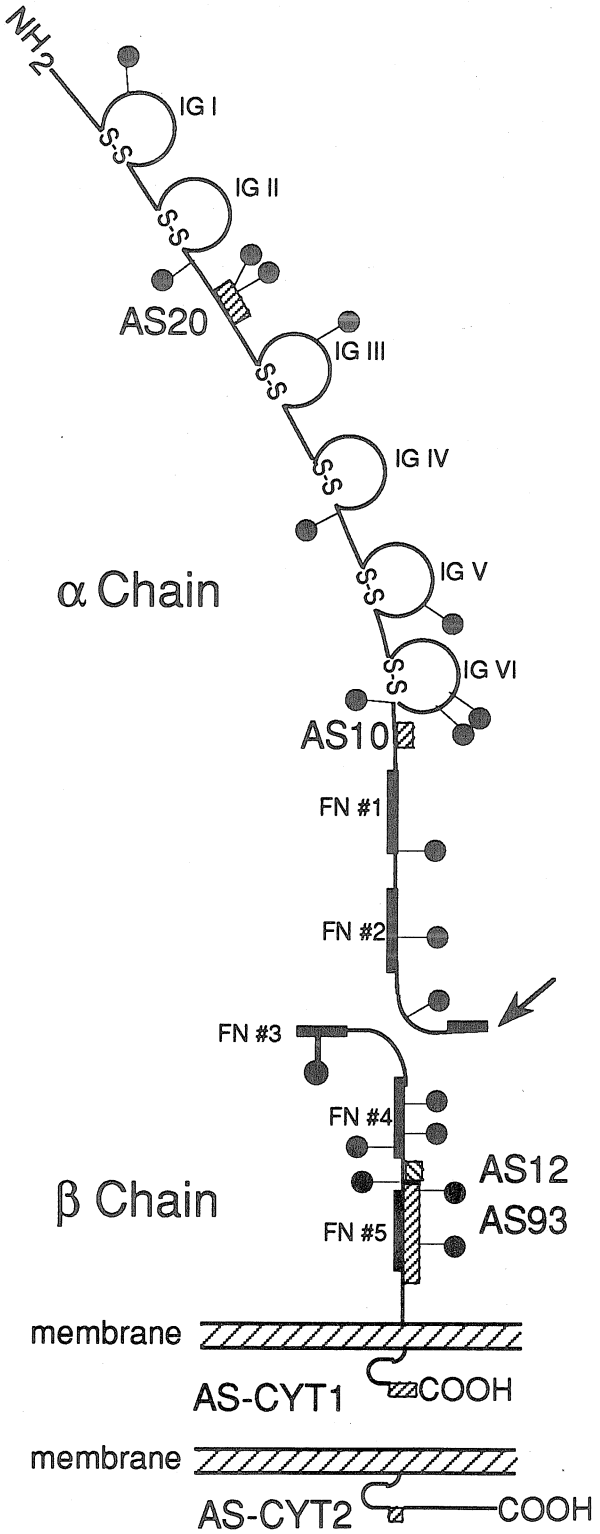
AS CYT2
BRAVO:
NgCAM:
L1:
BRAVO cont.:
NgCAM cont.:
L1 cont.:

Fig. 8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IG II
S-S
AS20
IG III
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IG IV
S-S
α Chain
IG V
S-S
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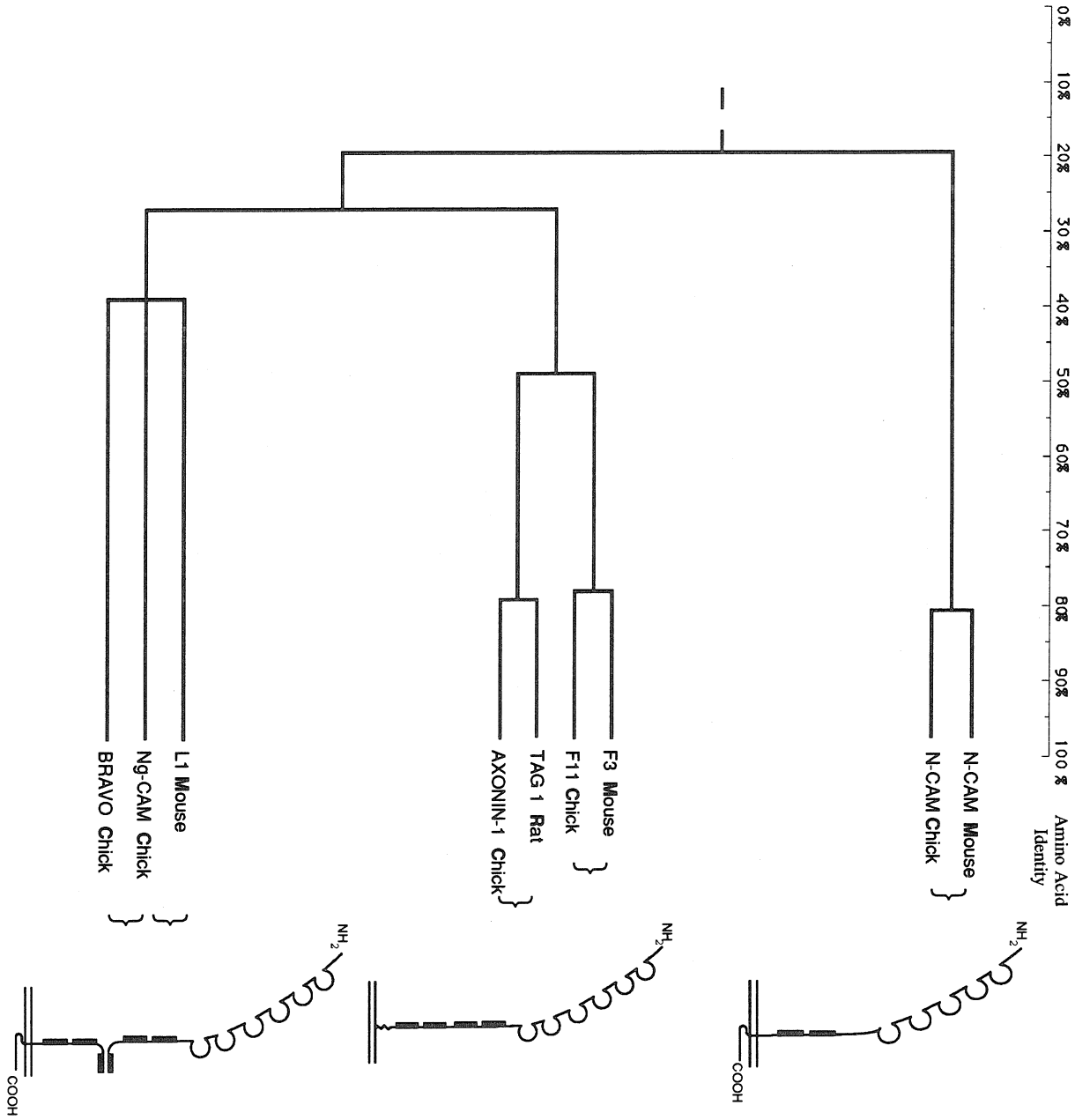
S-S
AS10
FN #1
FN #2
FN #3
FN #4
AS12
β Chain
AS93
FN #5
membrane
AS-CYT1
COOH
membrane
AS-CYT2
COOH

FIGURE 9


0%
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20%
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40%
50%
60%
70%
80%
90%
100 % Amino Acid Identity
N-CAM Mouse
N-CAM Chick
F3 Mouse
F11 Chick
TAG 1 Rat
AXONIN-1 Chick
L1 Mouse
Ng-CAM Chick
BRAVO Chick
NH2
COOH

Appendix I-1

Topologically Restricted Appearance in the Developing Chick Retinotectal System of Bravo, a Neural Surface Protein: Experimental Modulation by Environmental Cues

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Abstract. A novel neural surface protein, Bravo, shows a pattern of topological restriction in the embryonic chick retinotectal system. Bravo is present on the developing optic fibers in the retina; however, retinal axons in the tectum do not display Bravo. The appearance of Bravo in vitro is modulated by environmental cues. Axons growing out from retinal explants on retinal basal lamina, their natural substrate, express Bravo, whereas such axons growing on collagen do not. Retinal explants provide a valuable system to characterize the mechanism of Bravo restriction, as well as the cellular signals controlling it. Bravo was identified with monoclonal antibodies from a collection generated against exposed molecules isolated by using a selective cell surface biotinylation procedure. The NH_2-terminal sequence of Bravo shows similarity with L1, a neural surface molecule which is a member of the immunoglobulin superfamily. This possible relationship to L1, together with its restricted appearance, suggests an involvement of Bravo in axonal growth and guidance.

When neural networks are generated during embryogenesis, axons migrate along defined routes to their targets. The guidance of the axons depends on their interaction with the environment. Molecules exposed on the axonal surface and on the growth cone are assumed to participate in this interplay, a precondition of axonal outgrowth, fasciculation, and pathfinding (for reviews see Edelman, 1985; Jessell, 1988; Rathjen, 1988; Harris and Holt, 1990). An involvement of surface molecules in axonal outgrowth (Lagenaur and Lemmon, 1987; Bixby and Zhang, 1990; Chang et al., 1990; Furley et al., 1990), fasciculation (Rathjen et al., 1987*a*,*b*), and guidance (Matsunaga et al., 1988) has indeed been demonstrated. Many of these surface glycoproteins share structural features, such as the HNK-1 epitope (Pesheva et al., 1987; Gennarini et al., 1989) and immunoglobulin-like domains (Cunningham et al., 1987; Harrelson and Goodman, 1988; Moos et al. 1988; Ranscht, 1988; Seeger et al., 1988; Bieber et al., 1989; Brümmendorf et al., 1989; Gennarini et al., 1989; Furley et al., 1990). These domains supposedly are involved in cell adhesion and are indicators of the evolutionary development of cell interactions (Edelman, 1984, 1985, 1987; McClay and Ettensohn, 1987; Rutishauser et al., 1988; Schachner et al., 1988; Williams and Barclay, 1988).

Molecules involved in axonal guidance are expected to be expressed early in development, when neural connections are forming, under temporal or topological restriction (Raper et al., 1983*a*,*b*; Bastiani et al., 1984; Dodd and Jessell, 1988; Harrelson and Goodman, 1988). Indeed, fasciclins I and II in the grasshopper (Bastiani et al., 1987) and TAG-1 in the rat (Dodd et al., 1988) show a restricted pattern of expression. The mechanisms controlling such restricted expression are still not well understood.

The retinotectal system of the chick embryo is especially suited for studies of axonal outgrowth and navigation. Whole organ preparations, as well as retinal explant cultures, allow manipulation of the axonal environment under conditions that maintain the natural recognition properties of the optic fibers (Halfter et al., 1981; Bonhoeffer and Huf, 1982; Walter et al., 1987*a*,*b*).

This project was aimed at the identification and characterization of molecules involved in the interactions between axons and their environment, using monoclonal antibodies specifically made for this purpose. Optic tecta of chick embryos at day 8 of development (E8)[1] were chosen as a source of antigen. At this stage, the optic fibers massively invade the optic tectum but most have not reached their targets (Crossland et al., 1975; Rager, 1980). Thus, both inter-

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1. *Abbreviations used in this paper*: E8, embryonic day 8 of development; RT, room temperature.

active partners of the retinotectal system, the optic fibers and their tectal targets, are found together in a single structure. Assuming that molecules participating directly in cell interactions are located on the cell surface or in the extracellular matrix, exposed molecules in the optic tectum containing growing optic fibers were specifically biotinylated (Boxberg et al., 1990). The labeled molecules were isolated by avidin-affinity chromatography and further fractionated according to size by HPLC. mAbs were raised against each of the fractions, yielding a large collection of antibodies specific for surface molecules. A detailed description of the method will be presented (Kayyem, J. F., J. M. Roman, D. B. Teplow, E. J. de la Rosa, U. Schwarz, and W. J. Dreyer, manuscript in preparation). The molecules recognized by different mAbs were designated using the international phonetic alphabet (Alpha, Bravo, Charlie, etc.), since the Greek alphabet is already heavily used in naming other proteins.

Bravo is one of the neural surface compounds found with those mAbs. Some biochemical and biological characteristics of this molecule will be described in this article.

Materials and Methods

Chick Embryos

All the experiments were performed with White Leghorn embryos of the indicated ages, obtained by incubation of fertilized eggs at 38.7°C.

Immunochemical Methods

Purification of Antigen by Immunoaffinity Chromatography. Before its immobilization on a column, the antibody was purified from mouse ascites, either by HPLC on a TSK DEAE-5PW column (Bio-Rad Laboratories, Munich, Germany), according to Deschamps et al., 1985, or by chromatography on a CM Affi-Gel Blue column (Bio-Rad Laboratories), according to the manufacturer's instructions.

The purified mAb (25 mg) was bound to 10 ml of Affi-Gel 10 (Bio-Rad Laboratories), as recommended by the manufacturer, packed in a column which, before use, was run once with the various buffers to be used for antigen purification (see below).

An extract from P1 chick whole brain was used as an antigen source. Brains were washed three times with 3 ml per brain of labeling buffer (140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM $NaHCO_3$, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, and 0.5 μl/ml aprotinin, 2 mg/ml iodoacetamide, 0.2 mg/ml PMSF, and 50 μg/ml soybean trypsin inhibitor as protease inhibitors) and homogenized in 3 ml per brain of Nicholson lysis buffer (10 mM Hepes, pH 7.5, 140 mM NaCl, 4 mM EDTA, 2.5% (wt/vol) NP-40 (Calbiochem-Behring, Corp., Frankfurt am Main, Germany), 2.5% (wt/vol) Zwittergent 3-14 (Calbiochem-Behring Corp.) (Updyke and Nicholson, 1984) supplemented with azide and protease inhibitors (as above). After centrifugation (50,000 *g* for 30 min) the supernatant from 100 brains was pumped onto the column with immobilized antibody at a flow rate of 10 ml/h. The column was then washed at a flow rate of 50 ml/h with (*a*) 100 ml of 20 mM Tris-HCl, pH 8.0, containing 140 mM NaCl, 0.5% (wt/vol) NP-40, and 0.5% (wt/vol) Zwittergent 3-14; (*b*) 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.5% (wt/vol) NP-40; (*c*) 50 ml of 50 mM Tris-HCl, pH 9.0, containing 0.5 M NaCl and 0.1% (wt/vol) NP-40. The antigen was finally eluted with 150 ml of 50 mM triethanolamine, pH 11.5, containing 150 mM NaCl and 0.1% (wt/vol) NP-40. 1.6-ml fractions were collected in tubes containing 0.4 ml of 1 M Tris-HCl, pH 6.7. The antigen in the fractions was identified by a dot blot assay (see below). The antigen peak was pooled and concentrated-dialyzed by six cycles of centrifugation (1,500 *g* for 30 min) in Centriprep 30 (Amicon Corp., Witten, Germany), with PBS containing 0.1% (wt/vol) NP-40. The antigen was concentrated by a factor of 15 and the elution buffer was diluted 10^3 times. In the case of Bravo, the final concentration of antigen was 30 μg/ml, as determined by silver staining of SDS gels in comparison with standards. The final yield was 250–300 μg of protein from 100 brains. The column was regenerated by washing with 200 ml of 20 mM Tris-HCl, pH 8.0, containing 140 mM NaCl, and 0.025% (wt/vol) azide.

Protein Blots. For dot blots (Hawkes et al., 1982), 1–10 μl of the sample to be analyzed was spotted onto nitrocellulose and dried. To reduce background staining, the nitrocellulose was kept for at least 2 h at RT with 5% (wt/vol) powdered skim milk and washed twice with PBS before immunostaining. Hybridoma culture supernatant (1:4 dilution), mouse ascites (1:2,000 dilution), or rabbit antiserum (1:25,000 dilution) were incubated with the blot for 1 h at RT. For all dilutions, and three washings between incubations, PBS containing 0.1% (vol/vol) Tween 20 was used. Finally, the blots were incubated for 1 h at RT with a 1:1,000 dilution of peroxidase-coupled second antibody and stained with 4-chloronapthol (0.5 mg/ml) and 0.015% (vol/vol) of H_2O_2 in PBS.

For Western blots, the samples were fractionated by electrophoresis (SDS-PAGE; 6% gel) and transferred to nitrocellulose in a semi-dry system as described by Kyhse-Andersen (1984). The Western-blots were treated as described above for the dot-blots.

Protein Sequencing

NH_2-terminal protein sequence information was gathered using a protein sequenator (model 477A; Applied Biosystems, Foster City, CA) with on-line PTH analysis (model 120A; Applied Biosystems). Protein antigens (30–200 pmol), purified by affinity chromatography as described above, were electroblotted onto polyvinylide difluoride membrane after SDS-PAGE (Matsudaira, 1987).

Immunohistochemical Methods

Tissue Sections. Chick embryos were sectioned with a cryomicrotome after fixation of the tissue. For small embryos, up to E8, the standard fixation was by overnight immersion in 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. Larger embryos were first perfused with fixative (as above); then tissue pieces were dissected and further fixed by overnight immersion in fixative. Alternatively, the tissues were fixed by overnight immersion in 2% (wt/vol) TCA. Routinely, the fixed tissue was infiltrated with 30% (wt/vol) sucrose in PBS before sectioning.

For staining with single antibodies, the sections were incubated sequentially with (*a*) 15% (vol/vol) normal goat serum in culture medium for 20 min at RT: (*b*) mAb, hybridoma culture supernatant (undiluted), or mouse ascites (1:1,000 dilution) or rabbit polyclonal antiserum (1:20,000) for 45 min at RT: (*c*) biotinylated second antibody (anti-mouse or anti-rabbit of 1:200 dilution) for 30 min at RT: and (*d*) fluorescein-Streptavidin (1/200 dilution) for 30 min at RT. The washing steps between incubations, as well as the dilutions were with PBS containing 0.2% (wt/vol) BSA. The sections were mounted for epifluorescence microscopy.

For double staining, mAb anti-G4/L1 purified by HPLC (Deschamps et al., 1985) was labeled with biotin, according to Clark and Todd (1982). Biotin X-NHS (Calbiochem-Behring Corp.) as a freshly prepared 20 mg/ml solution in DMSO was added to the antibody in PBS (biotin/mAb; 1:7; wt/wt) and incubated for 2 h at RT with vigorous shaking. Excess biotin was reacted with a 10-fold excess of glycine (30 min at RT). The mixture was directly used for staining. Sections were double stained as follows: (*a*) 15% (vol/vol) normal goat serum for 20 min at RT; (*b*) mAb anti-Bravo (culture supernatant or a 1:1,000 dilution of mouse ascites) for 45 min at RT; (*c*) fluorescein-labeled anti-mouse second antibody (1:80 dilution) for 30 min at RT; (*d*) normal mouse serum (1 mg/ml) for 30 min at RT; (*e*) biotinylated mAb anti-G4/L1 (5 μg/ml) for 45 min at RT; and (*f*) Texas red-Streptavidin (1/100 dilution) for 30 min at RT. The stained sections were screened in an epifluorescence microscope, with either fluorescein or rhodamine filter.

Whole Mounts. Retinas removed from chicken eyes were flattened out on a filter, with the basal lamina (inner part) up, as described by Halfter et al. (1983). Tectal whole mounts were prepared by dissecting the tecta out of the rest of the brain and spreading the isolated tecta flat on a filter, with the ventricular surface (inner part) down, similar to the retinal whole mounts (Kröger and Schwarz, 1990).

For EM, E6 retinal whole mounts were pre-fixed in 4% (wt/vol) paraformaldehyde for 4 h at RT and stained as described for tissue sections, except that incubation and washing times were doubled and that, in the last step, the whole mounts were incubated with peroxidase-Streptavidin (1:100 dilution) for 1 h at RT and reacted with diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.02% vol/vol) in PBS with Co^{2+} and Ni^{2+} enhancement. The tissue was postfixed with 2.5% (vol/vol) glutaraldehyde for 1 h at RT and processed for EM.

Tectal whole mounts were stained with mAbs as described for tissue sections, but with doubled incubation times. For staining native tissue, the whole mounts were first incubated with the antibody, then fixed with 4% (wt/vol) paraformaldehyde for 2 h at RT, and then stained.

Explant Cultures. Retinal explant cultures (see below) were stained as

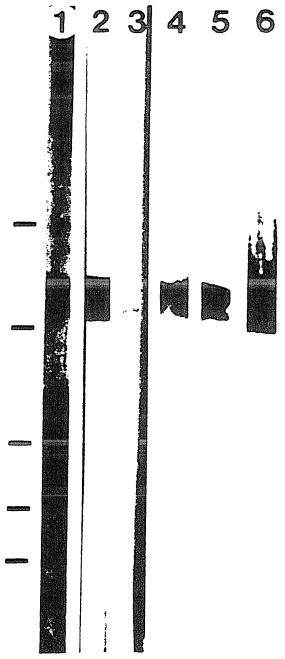


Figure 1. Biochemical characterization of Bravo. Shown are Western blots of immunoaffinity-purified Bravo antigen stained with either gold (lane *1*), mAb anti-Bravo (lane *2*), or mAb L2 (lane *3*). In extracts from E8 retina (lane *4*), E8 whole brain (lane *5*), and P1 whole brain (lane *6*), Bravo was identified by Western blot and immunostaining with mAb anti-Bravo. The antigen–antibody complexes were revealed using the peroxidase procedure. The marks at the left margin indicate the position of molecular mass standards of 180, 116, 84, 58, and 48.5 kD.

described for tissue sections. The tissue was fixed (30 min at RT with 4% paraformaldehyde (wt/vol) either before the incubation with the mAb, or after, when living explants had been stained.

Embryo Manipulations

Tracing of the Optic Fibers. The optic fibers were traced from the eye to the tectum by injection of rhodamine into the eye (Thanos and Bonhoeffer, 1983). A lateral window was opened in the shell of fertilized eggs after a 5-d incubation. The embryonic membranes were opened to allow access to the right eye, into which 1 μl of a freshly prepared rhodamine solution was injected (1 mg of rhodamine dissolved in 2 μl of DMSO, diluted with 100 μl PBS and clarified by centrifugation) with a glass capillary. After the manipulation, the window was covered with a petri dish (35 mm), sealed with silicone and further incubated for a minimum of 30 h before whole mount tecta (tectum contralateral of the injected eye) or tissue sections were prepared.

Eye Enucleation. Both eyes were removed from E2 chick embryos (Thanos and Dütting, 1988). 2 ml of the egg white was extracted from the egg with a syringe and a lateral window was opened to give access to the embryo, which was contrasted by injecting India ink underneath it. The optic vesicles were removed with tungsten needles and the egg was sealed with a petri dish cover (35 mm) and silicone and allowed to further develop in the incubator. At the desired stage, embryos were prepared for tissue sections.

Explant Cultures

Explant cultures from E6 retinae were prepared as described by Halfter et al. (1983). The retina, mounted flat on a membrane filter, was cut in strips with a tissue chopper set at 0.275 mm and cultured either on collagen gel or on retinal basal lamina, in DME/F12 medium (Gibco-BRL, Eggenstein, Germany) with 10% heat-inactivated FCS, 2% chick serum, and 50 μg/ml gentamicin. After 2 d in culture, the explants were stained. The collagen gel substrate was prepared from rat tail collagen, as described (Halfter et al., 1983). The basal laminae used as growth substrate were prepared from E7 retinae as described by Halfter et al. (1987). The basal lamina was fixed on petriperm plates (Heraeus-Amersil, Inc., Osterode, Germany), exposing the part which, in the retina, contacts the optic fibers. Such preparations, as shown by Halfter et al. (1987) consist of the basal lamina and the endfeet of ventricular cells.

Results

Biochemical Characterization of Bravo Antigen

Bravo antigen was purified by immunoaffinity chromatography. Two components of 130 and 140 kD on SDS-PAGE (Fig. 1) were isolated from a 1-d-old chick (P1) brain extract on a column with immobilized anti-Bravo antibody. On Western blots of retina and brain extracts, mAb anti-Bravo again stains two components of 130 and 140 kD. The amount of both components of the Bravo antigen (relative to wet weight of tissue) increases with the time of embryonic development (Fig. 1). Both the 130- and 140-kD components cross-react with the mAb L2, specific for the HNK-1 epitope found in other molecules involved in cell interactions (Schachner et al., 1988). The 130- and 140-kD molecules have the same NH_2-terminal sequence (Fig. 2), showing remarkable similarity to the L1 molecule, a member of a group of neural surface proteins that belongs to the immunoglobulin superfamily and contains fibronectin type III domains (Moos et al., 1988).

Bravo does not show immune cross-reactivity with the molecules G4, F11, or neurofascin (Rathjen et al., 1987*a*,*b*; Brümmendorf et al., 1989) (data not shown). This, together with the partial protein sequence data (Fig. 2), excludes identity between Bravo and other known molecules found in fiber-rich regions of the developing chick embryo.

Immunohistochemical Localization of Bravo in the Developing Chick Retinotectal System

mAb anti-Bravo stains all the fiber layers of the retina and the optic tectum as soon as they are morphologically identifiable. Bravo appears in parallel with G4, an early axonal marker which is considered to be the chick homologue of L1 (this molecule will be referred to here as G4/L1) (Rathjen et al., 1987*b*; Layer et al., 1988). Fig. 3 illustrates the simultaneous presence of both antigens at embryonic day 6 (E6), when the optic fiber fascicles in the retina (Fig. 3, *A* and *B*) and the circumferential fiber fascicles in the tectum (Fig. 3, *C* and *D*) are already well formed (LaVail and Cowan, 1971; Crossland et al., 1975; Puelles and Bendala, 1978; Rager, 1980). A coincident appearance of G4/L1 and Bravo

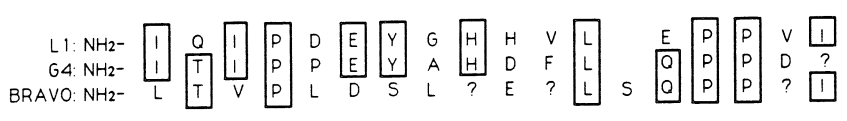


Figure 2. Comparison of the NH_2-terminal sequences of the axonal surface proteins L1, G4, and Bravo. The sequences of L1 and G4 have been taken from Moos et al. (1988) and Rathjen et al. (1987*b*), respectively. The sequence of Bravo was determined as described in Materials and Methods. Identical residues are squared. Note also that several nonidentical residues are functionally similar and suggest relatedness (e.g., isoleucine/leucine, glutamate/aspartate).

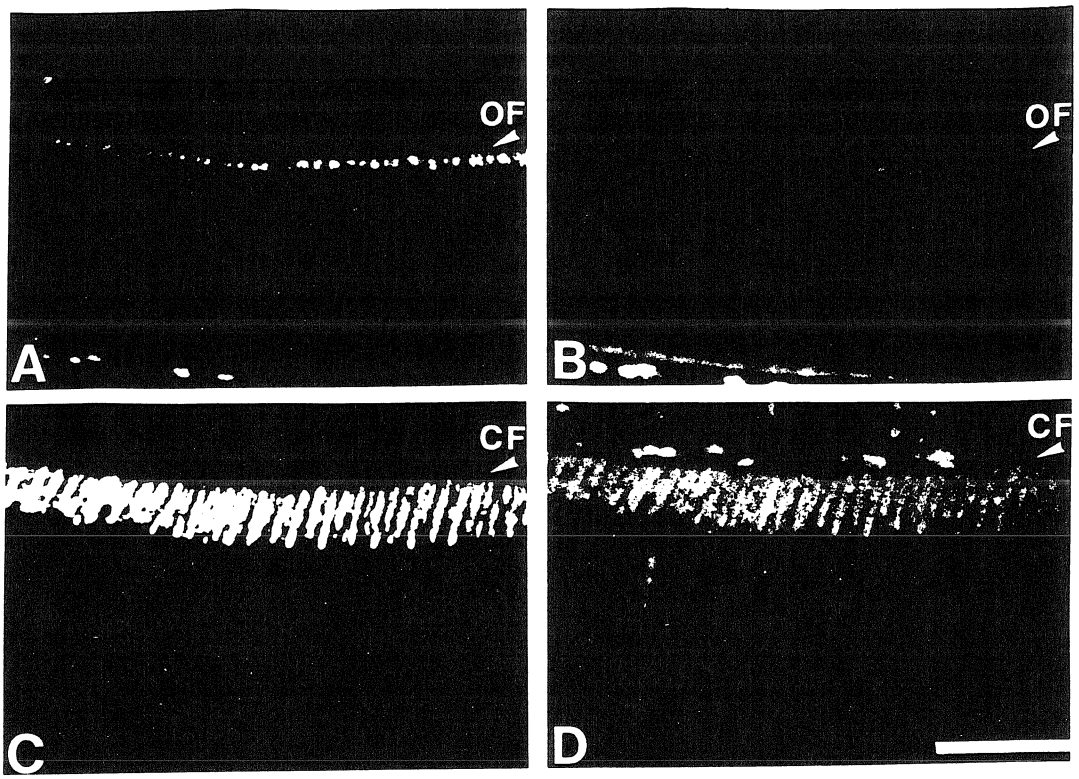


Figure 3. Parallel appearance of Bravo and G4/L1 in the developing chick retinotectal system. Shown are double-stained tissue sections of E6 chick embryo retina (*A* and *B*, same section) and tectum (*C* and *D*, same section). The axons are labeled either with mAb anti-G4/L1 (*A* and *C*) or with mAb anti-Bravo (*B* and *D*). Both the Bravo and the G4/L1 molecule appear in the first fibers originating in the retina, the optic fibers (*OF*) in *A* and *B*, and also in the first fibers that develop in the tectum, the circumferential fibers (*CF*) in *C* and *D*. Bar, 100 μm.

can be observed in the first emerging fibers along the whole neural tube and in cephalic vesicles at earlier stages of development (data not shown).

Since mAb anti-Bravo was raised against an antigen preparation enriched in surface molecules, we expected Bravo to be located on the cell surface or in the extracellular matrix. To investigate the precise location of this molecule, Bravo was also visualized in the retina by immunohistochemistry combined with EM. At E6, Bravo is clearly associated with the surface of the optic fibers in fascicles in the retina (Fig. 4). Furthermore, retinal explant cultures (see below) showed definitively that Bravo is exposed as an axonal surface molecule.

While the optic fibers in the retina carry Bravo, this molecule was not detected on retinal axons in the tectum. Optic fibers projecting to the tectum were traced by injection of rhodamine into the eye. The rhodamine-labeled fibers are visible when entering the tectum at the rostral pole of E6 whole mount tecta (Fig. 5, *A* and *C*). These fibers are not stained with mAb anti-Bravo (Fig. 5 *B*), but are stained with mAb anti-G4/L1 (Fig. 5 *D*). Circumferential fibers originating in the tectum, however, are stained by both mAbs (anti-G4/L1 and anti-Bravo), as shown by confocal microscopy of E6.5 tectal whole mounts (Fig. 6). By optical sectioning, the staining by mAbs anti-G4/L1 and anti-Bravo was analyzed throughout the depth of the tissue. Circumferential fibers in deeper layers carry G4/L1 as well as Bravo, in marked contrast to the optic fibers present in upper levels, only stained by mAb anti-G4/L1. Computer image processing of the pictures did not reveal, at any amplification of the signal, fiber structures in the top levels of the tectum stained with mAb anti-Bravo. In contrast, E6.5 whole mount retinas show clear staining of the optic fascicles with mAbs anti-Bravo as well as anti-G4/L1 (data not shown). Neither the optic fibers in the whole mount retinas nor the circumferential fibers in the whole mount tecta show regional differences in the level of Bravo. When considered together, all observations indicate that, while G4/L1 is present uniformly along the optic fibers, Bravo is detected differently in the retina than in the optic tectum. A polyclonal antiserum generated against Bravo yielded identical results (data not shown).

At E8, most of the optic fibers have already reached the tectum and are forming the *Stratum opticum* (Crossland et al., 1975; Rager, 1980). Again, the optic fibers were not stained with mAb anti-Bravo, neither in E8 whole mount tecta nor in E8 tectal sections (data not shown), which sup-

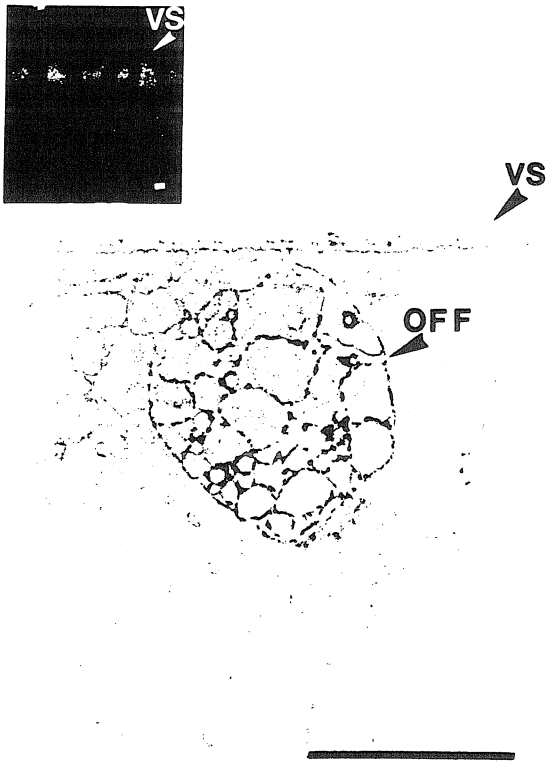


Figure 4. Localization of the Bravo molecule in the optic fibers in the retina. The electron micrograph shows an optic fiber fascicle (*OFF*) transversally sectioned in the central region of an E6 retina. Bravo surrounds the axons as revealed by immunostaining. The retina was stained as a whole mount with mAb anti-Bravo (peroxidase procedure) before being processed for EM. The inset shows the position of axon fascicles in an equivalent section, fluorescence-labeled with mAb anti-Bravo, in the light microscope. *VS* indicates the vitreal (inner) surface of the retina. Bars, 2.5 μm.

ports the observations made at earlier stages. On the contrary, the optic fibers were brightly stained by mAb anti-G4/L1 (data not shown). Consequently, the lack of staining by mAb anti-Bravo of the optic fibers in the tectum cannot result from a delayed expression of Bravo in comparison with G4/L1.

Bravo appears in the tectum in the same regions as the optic fibers, although these axons themselves are devoid of the Bravo molecule. To demonstrate that the appearance of Bravo in those optic regions is independent of the presence of optic fibers, tecta deprived of these fibers were studied. Fig. 7 shows sections of E8 tectum from embryos in which both eye vesicles had been removed at E2. These embryos lack the forming *Stratum opticum.* In contrast to the differences between normal and eyeless embryos stained for G4/L1 (Fig. 7, *A* and *C*), the general Bravo staining in the region of the forming *Stratum opticum* was not altered by the deprivation of fibers (Fig. 7, *B* and *D*). This suggests that tectal structures, other than optic fibers, react with the mAb anti-Bravo. Ventricular cell processes and end-feet present in that region are possible candidates. In support of this, preparations of pial and tectal-limiting membrane (Kröger and Schwarz, 1990), including ventricular cell end-feet but free of fibers, show a strong Bravo reactivity (data not shown).

Expression of Bravo on Axons of Retinal Explants

A topologically restricted appearance of Bravo in the optic fibers has been clearly shown. However, the morphological data do not give precise information about the molecular mechanisms underlying this phenomenon or the signals controlling it. A simple experimentally modifiable system was chosen for experiments to prove or disprove possible mechanisms and signals. Some of the early steps of neural development of the chick retinotectal system can be reproduced in explant cultures of embryonic retina (Halfter et al., 1981; Bonhoeffer and Huf, 1982; Halfter et al., 1983; Walter et al., 1987*a*,*b*). Therefore, this system was used to study the appearance of Bravo on axons growing on different substrates.

The basal lamina from the embryonic retina can be isolated, flattened out, and used as a substrate for outgrowing axons. This preparation consists of the inner basement membrane carrying the end feet of the ventricular cells (Halfter et al., 1987) and is very close to the natural substrate of optic fibers. Fibers grown on basal lamina indeed carry the Bravo molecule, as is the case in the embryonic retina (Fig. 8 *B*). The basal lamina preparation by itself does not show Bravo immunostaining. Intact living fibers are stained as well as fixed ones (data not shown), demonstrating the expected surface location of the Bravo molecule. This result was also reproduced with a polyclonal antiserum anti-Bravo (data not shown).

Most interestingly, however, the presence of Bravo on outgrowing retinal fibers was found to depend upon their environment. Very clearly, the fibers grown on collagen gel, which is a major structural component of the extracellular matrix but, in this case, not a natural substrate, carry very little, if any, Bravo (Fig. 8 *D*). Only close to the retina stripe, where some fibers fasciculate, a weak positive staining was detectable (data not shown). The lack of Bravo staining on fibers grown on collagen cannot result from an inaccessibility of the antibody to the fibers in the culture dish, since the mAb anti-G4/L1 stains them brightly (Fig. 8, *A* and *C*).

This result suggests that the appearance of Bravo on the surface of the optic fibers can be controlled by a signal associated with the retinal basal lamina, the natural substrate of these axons. The in vitro results are consistent with the in vivo situation, in which the optic fibers grow close to the basal lamina and display the Bravo molecule.

Discussion

Increasing evidence is emerging that indicates that some of the molecules involved in the interaction of the axon with its environment during axonal navigation are subject to change and modulation during embryogenesis. Patterns of expression of such molecules define particular regions of an axonal pathway. In the grasshopper, for example, fasciclins are differentially expressed on subsets of axon fascicles (Bastiani et al., 1987). Fasciclin I is exposed in particular commis-

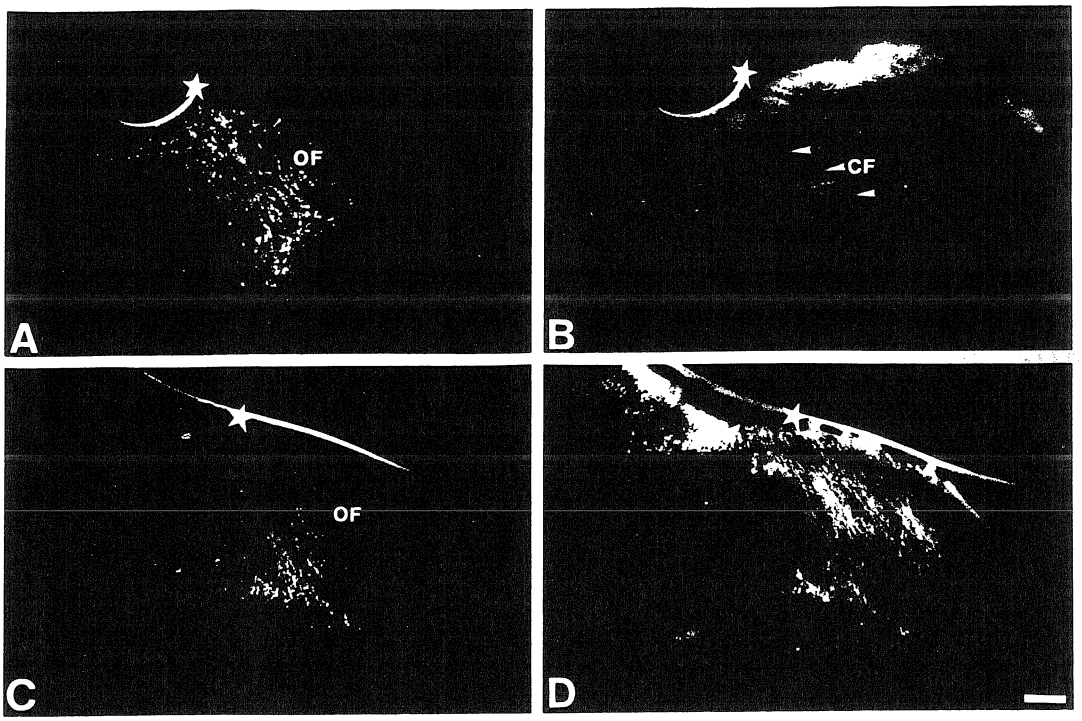
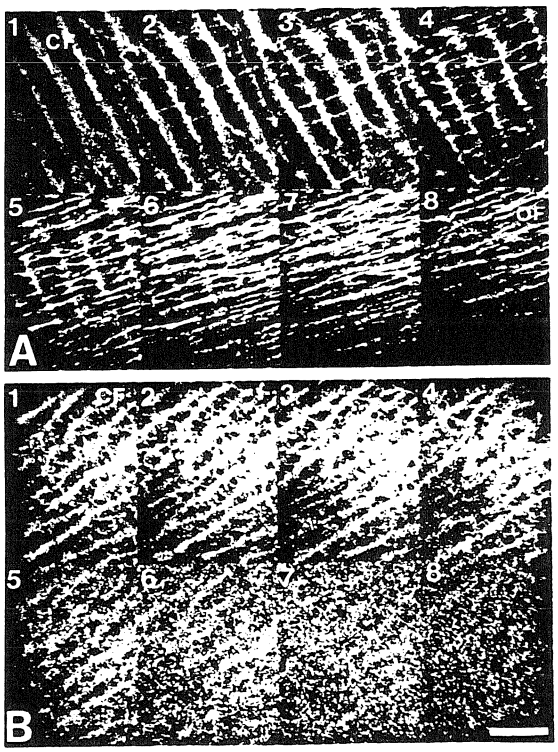


Figure 5. Absence of Bravo in the optic fibers when entering the tectum. Whole mount tecta showing rhodamine-labeled optic fibers (*OF*) in *A* and *C*, which are counterstained with mAb anti-Bravo (*B*), or with mAb anti-G4/L1 (*D*). Rhodamine was injected into the eye of E5 embryos. The subsequent staining with mAb anti-G4/L1 reveals a carpet of axons entering the tectum (*D*, same preparation as in *C*), which however is not detected with mAb anti-Bravo (*B*, same preparation as in *A*). Some tectal circumferential fibers in a deeper focal plane (*CF*) in *B* are stained with mAb anti-Bravo. The rostral poles of the tecta are indicated by stars. Bar, 100 μm.



sural pathways, whereas fasciclin I is replaced by fasciclin II in longitudinal axon bundles. Quite similarly, in the rat embryo the glycoprotein TAG-1 appears transiently on subsets of spinal cord axons in commissural processes, whereas longitudinal fascicles express the molecule L1 (identical or closely related to Ng-CAM, NILE, G4/L1, and 8D9) (Dodd et al., 1988; Rathjen, 1988). The replacement of fasciclin I by fasciclin II, and of TAG-1 by L1, occurs in a single axon that expresses different proteins in defined regions of its pathway.

In addition to axonal surface protein shifts, the posttranslational modification of a single protein along axonal pathways has been observed. In the chick embryo, between days E5 and E10, the embryonic form of N-CAM, which is highly sialylated and weakly adhesive, as well as its adult form, less sialylated and highly adhesive, appear in parallel on different stretches along the trajectory of optic fibers (Schlosshauer et al., 1984). Whereas the perikaryon and intraretinal axons

Figure 6. Presence of Bravo and G4/L1 in the developing optic tectum. Whole mount tecta are shown, "optically sectioned" with a confocal microscope (system of Bio-Rad Laboratories, coupled with a Zeiss inverted microscope). The tecta (E6.5), labeled with mAb anti-G4/L1 (*A*) or with mAb anti-Bravo (*B*), were scanned (every 3 μm) from the bottom (section *1*) to the top (section *8*). The circumferential fascicles (*CF*) in deeper layers (*1–4*) are stained with both antibodies whereas the optic fibers (*OF*) in the upper layers (*5–8*), readily stained with mAb anti-G4/L1, are not detected by mAb anti-Bravo staining. Bar, 100 μm.

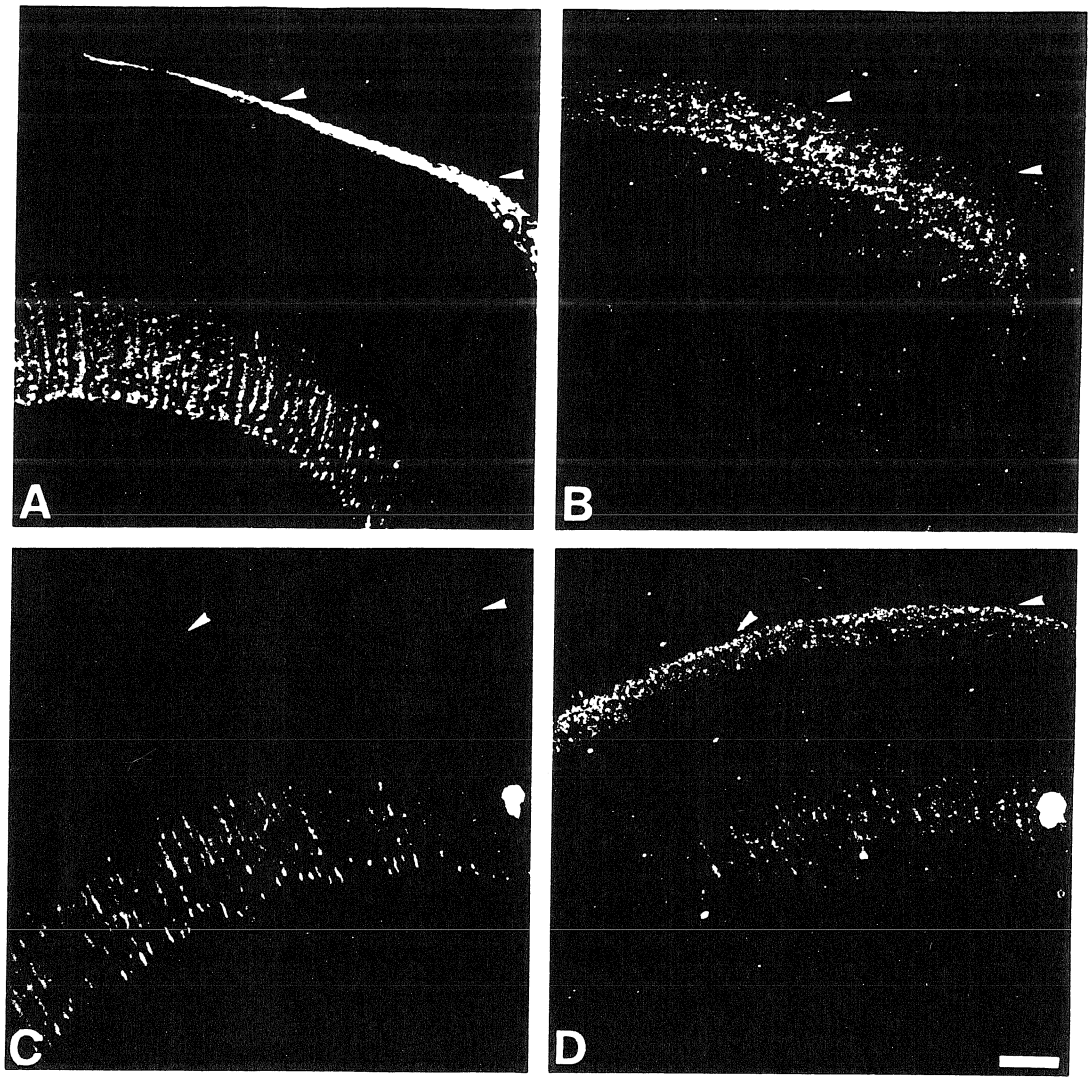


Figure 7. Presence of the Bravo molecule in the tectum deprived of optic fibers. Tectal sections are shown of a normal E8 embryo (*A* and *B*, same section), compared with those of an eyeless E8 embryo (*C* and *D*, same section), from which both optic vesicles had been removed at E2. The sections were stained with mAb anti-G4/L1 (*A* and *C*), which demonstrates the complete absence of optic fibers (*OF* in *A*) in the eyeless embryo (*C*). mAb anti-Bravo (*B* and *D*) reveals the presence of the Bravo molecule also in tecta devoid of optic fibers (*D*), indicating that Bravo is present in structures other than the optic fibers. A slight difference observed between normal and optic fiber–deprived tecta is due to the lag in development as a consequence of the manipulation during enucleation. The arrows indicate the position of the forming *Stratum opticum* in *A* and *B* and the corresponding position in *C* and *D*. Bar, 100 μm.

carry the low sialic acid derivative, extraretinal axons expose the embryonic form rich in sialic acid.

As described here, another case of spatial restriction of surface compounds along axonal pathways is reflected by the appearance of the Bravo molecule along the retinotectal projection. Bravo appears as a surface protein early in development, at about the time when axons start to emerge from neuronal cells. Optic fibers within the retina expose the Bravo molecule (Figs. 3 *B* and 4), whereas, at the same stage of development, when entering the tectum, these axons are devoid of Bravo or carry it in an undetectable form (Figs. 5 and 6). As opposed to G4/L1, whose distribution is relatively uniform along the optic fibers, Bravo shows a topologically restricted location in the chick retinotectal projection.

The mechanisms underlying the topologically restricted expression of molecules are not yet well characterized. The expression of TAG-1/L1 and of fasciclins I and II may be controlled either by an autonomous program of the neuron or by signals from the axonal environment. In the case of Bravo, the appearance of the molecule on the axonal surface de-

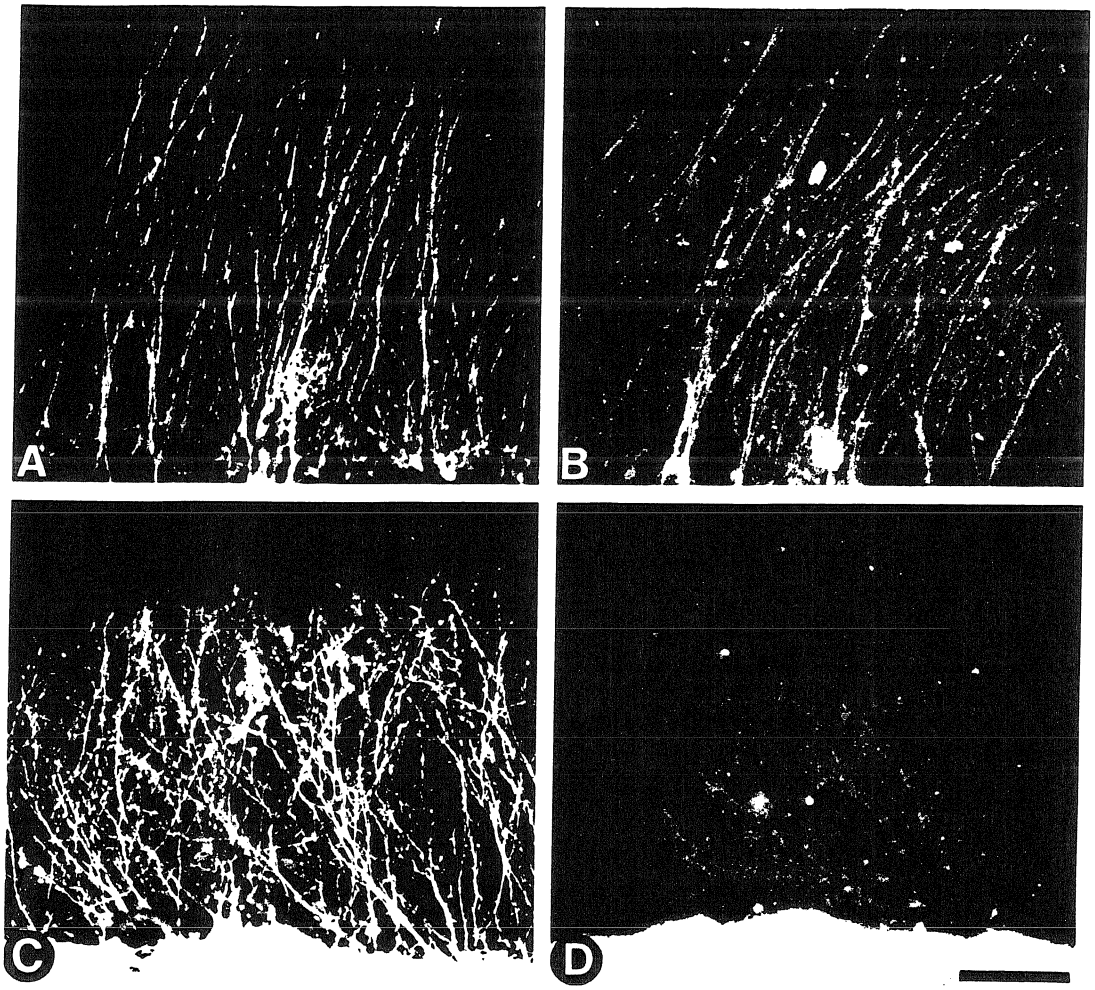


Figure 8. Substrate-dependent appearance of Bravo on fibers in retinal explant cultures. Shown are retinal explants grown for 2 d on retinal basal lamina (*A* and *B*) or on collagen gel (*C* and *D*). The explants were stained with mAb anti-G4/L1 (*A* and *C*) or with mAb anti-Bravo (*B* and *D*). The staining with mAb anti-G4/L1 is intense on fibers grown on either substrates (*A* and *C*). On the contrary, fibers grown only on retinal basal lamina are intensively stained with mAb anti-Bravo (*B*), in contrast to fibers grown on collagen (*D*). The explanted retina stripes are at the bottom of the pictures. The preparations of each mAb were photographed and printed under identical exposure conditions. Bar, 100 μm.

pends, at least in vitro, on environmental cues. Axons grown on retinal basal lamina carry Bravo, in contrast to axons grown on collagen gel (Fig. 8). The precise nature of the environmental signals, of their topological control, and of the cellular response remains to be elucidated.

Many mechanisms could account for such a pattern of topological restriction. Differential synthesis and turnover, selective membrane insertion and stabilization by interaction with cytoskeletal or extracellular components, and differential release from the membrane are, in principle, equally plausible. The simplicity of the retinal explant culture system, as described in this paper, allows manipulations of the fiber environment and observation of its consequences on the expression of the Bravo molecule. The availability of such a system is a prerequisite to analyze the cellular and molecular mechanisms involved in topological restriction. In addition, confocal microscopy together with image processing and analysis should allow a precise quantification of the amounts of Bravo.

The Bravo molecule is also found in neural tracts other than the optic fibers. This implies a role of the molecule beyond the retinotectal system. At present, a discussion of the function of Bravo has to remain speculative. The restricted pattern of expression during the formation of the retinotectal projection suggests a role in axonal navigation. Also the possible relationship of Bravo with L1 and with other members of the immunoglobulin superfamily, signals its involvement in cell-cell and/or fiber-fiber contacts. The possibility that

Bravo functions through a specific binding interaction with itself, with G4/L1, or with other molecules needs to be investigated.

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References

Bastiani, M. J., J. A. Raper, and C. S. Goodman. 1984. Pathfinding by neuronal growth cones in grasshopper embryos. III. Selective affinity of the G growth cone for the P cells within the A/P fascicle. *J. Neurosci.* 4:2311–2328.

Bastiani, M. J., A. L. Harrelson, P. M. Snow, and C. S. Goodman. 1987. Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell.* 48:745–755.

Bieber, A. J., P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. 1989. Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell.* 59:447–460.

Bixby, J. L., and R. Zhang. 1990. Purified N-Cadherin is a potent substrate for the rapid induction of neurite outgrowth. *J. Cell Biol.* 110:1253–1260.

Bonhoeffer, F., and J. Huf. 1982. *In vitro* experiments on axon guidance demonstrating an anterior-posterior gradient on the tectum. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:427–431.

Boxberg, Y. von, R. Wutz, and U. Schwarz. 1990. Use of the biotin-avidin system for the labeling, isolation and characterization of neural cell surface proteins. *Eur. J. Biochem.* 190:249–256.

Brümmendorf, T., J. M. Wolff, R. Frank, and F. G. Rathjen. 1989. Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. *Neuron.* 2:1351–1361.

Chang, S., F. G. Rathjen, and J. A. Raper. 1990. Neurite outgrowth promoting activity of G4 and its inhibition by monoclonal antibodies. *J. Neurosci. Res.* 25:180–186.

Clark, B. R., and C. W. Todd. 1982. Avidin as a precipitant for biotin-labeled antibody in a radioimmunoassay for carcinoembryonic antigen. *Anal. Biochem.* 121:257–262.

Crossland, W. J., W. M. Cowan, and L. A. Rogers. 1975. Studies on the development of the chick optic tectum. IV. An autoradiographic study of the development of the retinotectal connections. *Brain Res.* 91:1–23.

Cunningham, B. A., J. J. Hemperly, B. A. Murray, E. A. Prediger, R. Brackenbury, and G. M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation and alternative RNA splicing. *Science (Wash. DC.).* 236:799–806.

Deschamps, J. F., J. E. K. Hildreth, D. Derr, and J. T. August. 1985. A high-performance liquid chromatographic procedure for the purification of mouse monoclonal antibodies. *Anal. Biochem.* 147:451–454.

Dodd, J., and T. M. Jessell. 1988. Axon guidance and patterning of neuronal projections in vertebrates. *Science (Wash. DC.).* 242:692–699.

Dodd, J., S. B. Morton, D. Karagogeos, M. Yamamoto, and T. M. Jessell. 1988. Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron.* 1:105–116.

Edelman, G. M. 1984. Modulation of cell adhesion during induction, histogenesis and perinatal development of the nervous system. *Annu. Rev. Neurosci.* 7:339–377.

Edelman, G. M. 1985. Cell adhesion and the molecular processes of morphogenesis. *Annu. Rev. Biochem.* 54:145–169.

Edelman, G. M. 1987. CAMs and Igs: cell adhesion and the evolutionary origins of immunity. *Immunol. Rev.* 100:11–45.

Furley, A. J., S. B. Morton, D. Manalo, D. Karagogeos, J. Dodd, and T. M. Jessell. 1990. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell.* 61:157–170.

Gennarini, G., G. Cibelli, G. Rougon, M.-G. Mattei, and C. Goridis. 1989. The mouse neuronal cell surface protein F3: a phosphatidylinositol-anchored member of the immunoglobulin superfamily related to chicken contactin. *J. Cell Biol.* 109:775–788.

Gennarini, G., G. Rougon, F. Vitiello, P. Corsi, C. Di Benedetta, and C. Goridis. 1989. Identification and cDNA cloning of a new member of the L2/HNK-1 family of neural surface glycoproteins. *J. Neurosci. Res.* 22:1–12.

Halfter, W., M. Claviez, and U. Schwarz. 1981. Preferential adhesion of tectal membranes to anterior embryonic chick retina neurites. *Nature (Lond.).* 292:67–70.

Halfter, W., D. F. Newgreen, J. Sauter, and U. Schwarz. 1983. Oriented axon outgrowth from avian embryonic retinae in culture. *Dev. Biol.* 95:56–64.

Halfter, W., W. Reckhaus, and S. Kröger. 1987. Nondirected axonal growth on basal lamina from avian embryonic neural retina. *J. Neurosci.* 7:3712–3722.

Harrelson, A. L., and C. S. Goodman. 1988. Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. *Science (Wash. DC).* 242:700–708.

Harris, W. A., and C. E. Holt. 1990. Early events in the embryogenesis of the vertebrate visual system: cellular determination and pathfinding. *Annu. Rev. Neurosci.* 13:155–169.

Hawkes, R., E. Niday, and J. Gordon. 1982. A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* 119:142–147.

Jessel, T. M. 1988. Adhesion molecules and the hierarchy of neural development. *Neuron.* 1:3–13.

Kröger S., and U. Schwarz. 1990. The avian tectobulbar tract: development, explant culture and effects of antibodies on the pattern of neurite outgrowth. *J. Neurosci.* In press.

Kyhse-Andersen, J. 1984. Electrobloting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods.* 10:203–209.

Lagenaur, C., and V. Lemmon. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. USA.* 84:7753–7757.

LaVail, J. H., and W. M. Cowan. 1971. The development of the chick optic tectum. I. Normal morphology and cytoarchitectonic development. *Brain Res.* 28:391–419.

Layer, P. G., R. Alber, and F. G. Rathjen. 1988. Sequential activation of butyrylcholinesterase in rostral half somites and acetylcholinesterase in motoneurones and myotomes preceding growth of motor axons. *Development.* 102:387–396.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidine difluoride membranes. *J. Biol. Chem.* 262: 10035–10038.

Matsunaga, M., K. Hatta, A. Nagafuchi, and M. Takeichi. 1988. Guidance of optic nerve fibers by N-cadherin adhesion molecules. *Nature (Lond.).* 334:62–64.

McClay, D. R., and C. A. Ettensohn. 1987. Cell adhesion in morphogenesis. *Annu. Rev. Cell. Biol.* 3:319–345.

Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Früh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature (Lond.).* 334:701–703.

Pesheva, P., A. F. Horwitz, and M. Schacher. 1987. Integrin, the cell surface receptor for fibronectin and laminin, expresses the L2/HNK-1 and L3 carbohydrate structures shared by adhesion molecules. *Neurosci. Lett.* 83:303–306.

Puelles, L., and M. C. Bendala. 1978. Differentiation of neuroblasts in the chick optic tectum up to eight days of incubation: a Golgi study. *Neuroscience.* 3:307–325.

Rager, G. H. 1980. Development of the retinotectal projection in the chicken. *In* Advances in Anatomy, Embryology and Cell Biology 63. A. Brodal et al., editors. Springer-Verlag, Berlin/Heidelberg/New York.

Ranscht, B. 1988. Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. *J. Cell Biol.* 107:1561–1573.

Raper, J. A., M. Bastiani, and C. S. Goodman. 1983*a*. Pathfinding by neuronal growth cones in grasshopper embryos. I. Divergent choices made by the growth cones of sibling neurons. *J. Neurosci.* 3:20–30.

Raper, J. A., M. Bastiani, and C. S. Goodman. 1983*b*. Pathfinding by neuronal growth cones in grasshopper embryos. II. Selective fasciculation onto specific axonal pathways. *J. Neurosci.* 3:31–41.

Rathjen, F. G. 1988. A neurite outgrowth-promoting molecule in developing fiber tracts. *Trends Neurosci.* 11:183–184.

Rathjen, F. G., J. M. Wolff, S. Chang, F. Bonhoeffer, and J. A. Raper. 1987*a*. Neurofascin: a novel chick cell-surface glycoprotein involved in neurite-neurite interactions. *Cell.* 51:841–849.

Rathjen, F. G., J. M. Wolff, R. Frank, F. Bonhoeffer, and U. Rutishauser. 1987*b*. Membrane glycoproteins involved in neurite fasciculation. *J. Cell*

Biol. 104:343–353.

Rutishauser, U., A. Acheson, A. K. Hall, D. M. Mann, and J. Sunshine. 1988. The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science (Wash. DC.).* 240:53–57.

Schachner, M., H. Antonicek, T. Fahrig, A. Faissner, G. Fischer, V. Künemund, R. Martini, A. Meyer, E. Persohn, E. Pollerberg, R. Probstmeier, K. Sadoul, R. Sadoul, B. Seilheimer, and G. Thor. 1988. Families of neural cell adhesion molecules. *In* The Cell in Contact II: Structure and Function of Adhesion Molecules. G. M. Edelman, and J.-P. Thiery, editors. John Wiley & Sons Inc. New York. 443–468.

Schlosshauer, B., U. Schwarz, and U. Rutishauser. 1984. Topological distribution of different forms of N-CAM in the developing chick visual system. *Nature (Lond.).* 310:141–143.

Seeger, M. A., L. Haffley, and T. C. Kaufman. 1988. Characterization of *amalgam*: a member of the immunoglobulin superfamily from Drosophila. *Cell.* 55:589–600.

Thanos, S., and F. Bonhoeffer. 1983. Investigations on the development and topographic order of retinotectal axons: anterograde and retrograde staining of axons and perikarya with rhodamine *in vivo*. *J. Comp. Neurol.* 219:420–430.

Thanos, S., and D. Dütting. 1988. Plasticity in the developing chick visual system: topography and maintenance of experimentally induced ipsilateral projections. *J. Comp. Neurol.* 278:303–311.

Updyke, T. V., and G. L. Nicholson. 1984. Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices. *J. Immunol. Methods.* 73:83–95.

Walter, J., B. Kern-Veits, J. Huf, B. Stolze, and F. Bonhoeffer. 1987*a*. Recognition of position-specific properties of tectal cell membranes by retinal axons *in vitro*. *Development (Camb.).* 101:685–696.

Walter, J., S. Henke-Fahle, and F. Bonhoeffer. 1987*b*. Avoidance of posterior tectal membranes by temporal retinal axons. *Development (Camb.).* 101: 909–913.

Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily-domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381–405.

A Retinal Cell Surface Protein Unequally Distributed on Optic Fibers

The proper establishment of projections in the nervous system may be achieved by an address system of cell surface proteins. These address molecules are expected to be distributed unevenly temporally and spatially. One candidate for such a molecule was detected with the monoclonal antibody 10/22A8 produced as described in Chapter III. In the chick retina at embryonic stages E7 to E8, 10/22A8 stains the optic fibers of the nasal half of the retina more intensely than the fibers of the temporal half. This might be due to the fact that the differentiation of the temporal retina is more advanced and therefore has already down-regulated the expression of this molecule. Alternatively the unequal distribution of this protein could reflect the unequal distribution of an address molecule marking nasal fibers differently than temporal fibers. Horizontal sections through the optic chiasm reveal preferential staining of nasal fibers in the optic nerve (Fig. 1). In addition, 10/22A8 stains the cerebellum in a very restricted pattern (Fig. 2).

Sequencing of a cDNA clone containing a portion of the gene coding for this protein has revealed that it contains multiple C2 Ig-like domains and fibronectin type III repeats, but is not identical to any known chicken molecule. It appears to be most closely related to LAR (leukocyte antigen related molecule from man) and DLAR (a neuron-specific similar molecule from *drosophila*), two members of a family of membrane bound tyrosine phosphatases. Cloning and sequencing of the entire coding region will reveal whether this is in fact a novel member of the tyrosine phosphatase family and will help in generating epitope specific antibodies to investigate the function of this molecule.

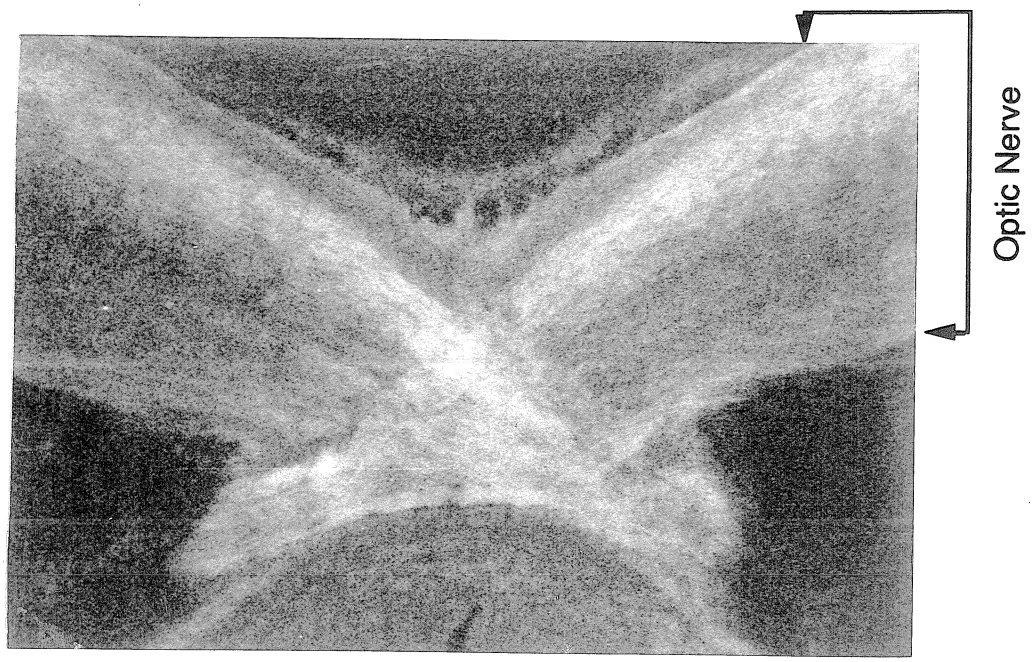

Fig. 1-Optic Nerve and Chiasm Stained with 10/22A8

Horizontal section through the E8 optic nerves and chiasm fluorescence labeled with MAb 10/22A8. Note the bright staining of only a distinct subset of axons in the optic nerve. Similarly labeled retinal sections reveal this localization to be primarily nasal and not temporal fibers.

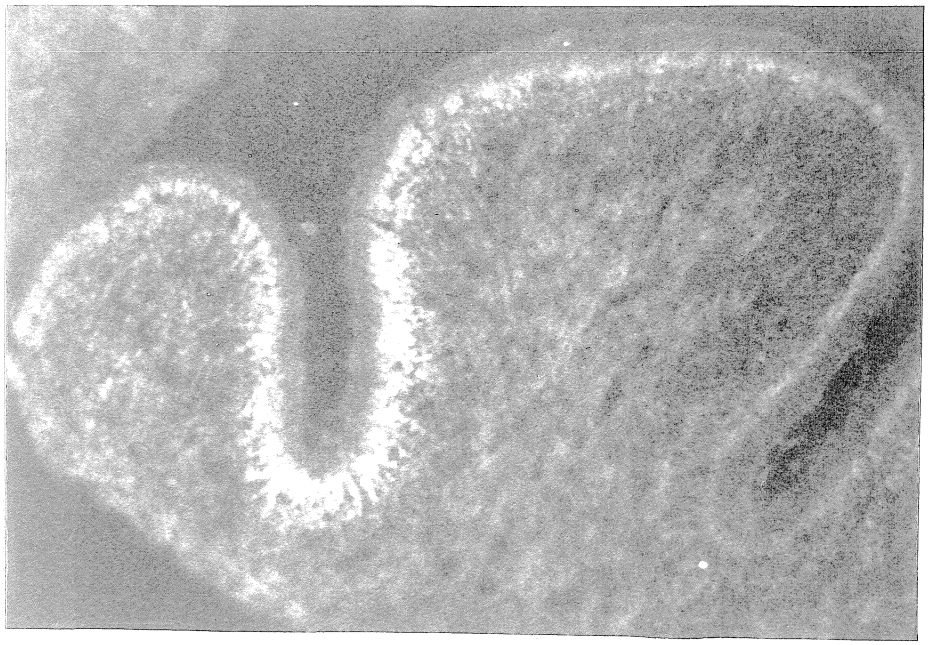

Fig. 2-Cerebellum stained with 10/22A8

Sagittal section through E10 cerebellu: fluorescence labeled with MAb 10/22A8 showing only the two most posterior foliae. Note the bright staining in the external granular cell layer which fades away in the second folium. Although not shown, no other folia are stained by this MAb.

Appendix III--Nucleotide and Deduced Amino Acid Sequence of Bravo

```
                    ①                                                  Ig I          +
 36   V  I  Q  C  E  A  K  G  K  P  P  P  S  F  S  W  T  R  N  G                      55
106   GTAATACAATGTGAAGCAAAAGGAAAACCACCTCCTAGCTTCTCCTGGACGCGCAATGGA                    165

 56   T  H  F  D  I  D  K  D  A  Q  V  T  M  K  P  N  S  G  T  L                      75
166   ACTCATTTTGATATAGATAAAGATGCACAGGTAACAATGAAACCAAATTCAGGAACCCTT                    225
                                                                    ❶
 76   V  V  N  I  M  N  G  V  K  A  E  A  Y  E  G  V  Y  Q  C  T                      95
226   GTTGTAAATATTATGAATGGTGTGAAGGCAGAAGCATATGAAGGAGTATACCAGTGTACA                    285

 96   A  R  N  E  R  G  A  A  I  S  N  N  I  V  I  R  P  S  R  S                     115
286   GCAAGGAATGAAAGAGGAGCAGCCATTTCCAACAATATTGTTATACGGCCATCTAGATCC                    345

116   P  L  W  T  K  E  K  L  E  P  N  H  V  R  E  G  D  S  L  V                     135
346   CCTTTGTGGACTAAAGAAAAACTAGAACCAAATCATGTTCGAGAAGGTGATTCCCTAGTA                    405
         ②                                                  Ig II
136   L  N  C  R  P  P  V  G  L  P  P  P  I  I  F  W  M  D  N  A                     155
406   CTAAACTGCAGACCTCCTGTTGGCTTACCACCACCTATAATATTTTGGATGGATAATGCT                     465

156   F  Q  R  L  P  Q  S  E  R  V  S  Q  G  L  N  G  D  L  Y  F                     175
466   TTCCAAAGGCTGCCTCAAAGTGAAAGAGTTTCTCAAGGTCTCAATGGAGACCTTTATTTT                    525
                                    ❷                              +
176   S  N  V  Q  P  E  D  T  R  E  D  Y  I  C  Y  A  R  F  N  H                     195
526   TCTAATGTACAACCAGAGGACACCCGTGAGGACTATATCTGCTACGCGAGATTTAATCAC                     585

196   T  Q  T  I  Q  Q  K  Q  P  I  S  V  K  V  F  S  T  K  P  V                     215
586   ACACAAACTATACAGCAGAAACAACCCATTTCTGTAAAAGTCTTTTCAACCAAGCCAGTT                     645

216   T  E  R  P  P  V  L  L  T  P  M  G  S  T  S  N  K  V  E  L                     235
646   ACAGAAAGGCCACCAGTTCTTCTTACACCAATGGGCAGCACAAGTAACAAAGTGGAACTG                     705
                        ③
236   R  G  N  V  L  L  L  E  C  I  A  A  G  L  P  T  P  V  I  R                     255
706   AGAGGAAATGTTCTTTTGTTGGAATGCATTGCAGCAGGATTACCCACACCAGTAATCCGC                     765
Ig III                  +
256   W  I  K  E  G  G  E  L  P  A  N  R  T  F  F  E  N  F  K  K                     275
766   TGGATTAAAGAGGGTGGTGAACTGCCAGCCAACAGAACGTTTTTTGAAAATTTTAAGAAA                    825
                                                                ❸
276   T  L  K  I  I  D  V  S  E  A  D  S  G  N  Y  K  C  T  A  R                     295
826   ACTCTCAAGATTATAGACGTCTCTGAAGCTGACTCTGGGAACTACAAATGTACAGCAAGA                     885

296   N  T  L  G  S  T  H  H  V  I  S  V  T  V  K  A  A  P  Y  W                     315
886   AATACATTGGGTTCTACTCATCATGTCATTTCGGTAACTGTAAAAGCTGCCCCATACTGG                     945
                                                                ④
316   I  T  A  P  R  N  L  V  L  S  P  G  E  D  G  T  L  I  C  R                     335
946   ATAACAGCACCCAGGAACTTAGTATTGTCTCCTGGAGAAGATGGGACATTGATCTGCAGA                    1005
                                 Ig IV
336   A  N  G  N  P  K  P  S  I  S  W  L  T  N  G  V  P  I  A  I                     355
1006  GCTAATGGCAACCCAAAACCTAGCATAAGCTGGTTAACAAATGGCGTTCCCATAGCAATT                    1065

356   A  P  E  D  P  S  R  K  V  D  G  D  T  I  I  F  S  A  V  Q                     375
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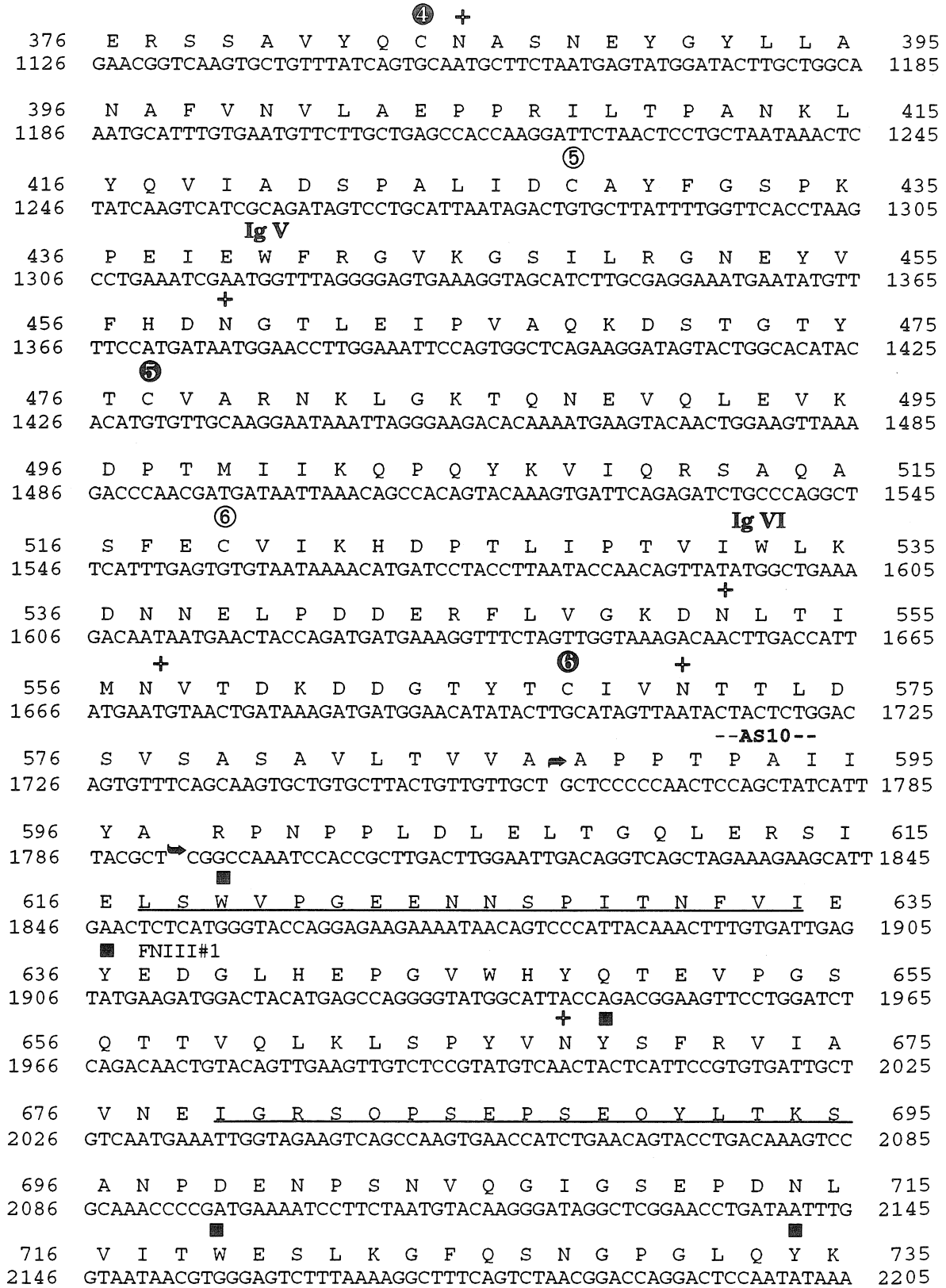
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396 N A F V N V L A E P P R I L T P A N K L 415
1186 AATGCATTTGTGAATGTTCTTGCTGAGCCACCAAGGATTCTAACTCCTGCTAATAAACTC 1245
⑤
416 Y Q V I A D S P A L I D C A Y F G S P K 435
1246 TATCAAGTCATCGCAGATAGTCCTGCATTAATAGACTGTGCTTATTTTGGTTCACCTAAG 1305
Ig V
436 P E I E W F R G V K G S I L R G N E Y V 455
1306 CCTGAAATCGAATGGTTTAGGGGAGTGAAAGGTAGCATCTTGCGAGGAAATGAATATGTT 1365
+
456 F H D N G T L E I P V A Q K D S T G T Y 475
1366 TTCCATGATAATGGAACCTTGGAAATTCCAGTGGCTCAGAAGGATAGTACTGGCACATAC 1425
⑤
476 T C V A R N K L G K T Q N E V Q L E V K 495
1426 ACATGTGTTGCAAGGAATAAATTAGGGAAGACACAAAATGAAGTACAACTGGAAGTTAAA 1485
496 D P T M I I K Q P Q Y K V I Q R S A Q A 515
1486 GACCCAACGATGATAATTAAACAGCCACAGTACAAAGTGATTCAGAGATCTGCCCAGGCT 1545
⑥
Ig VI
516 S F E C V I K H D P T L I P T V I W L K 535
1546 TCATTTGAGTGTGTAATAAAACATGATCCTACCTTAATACCAACAGTTATATGGCTGAAA 1605
+
536 D N N E L P D D E R F L V G K D N L T I 555
1606 GACAATAATGAACTACCAGATGATGAAAGGTTTCTAGTTGGTAAAGACAACTTGACCATT 1665
+ ⑥ +
556 M N V T D K D D G T Y T C I V N T T L D 575
1666 ATGAATGTAACTGATAAAGATGATGGAACATATACTTGCATAGTTAATACTACTCTGGAC 1725
--AS10--
576 S V S A S A V L T V V A ⇌ A P P T P A I I 595
1726 AGTGTTTCAGCAAGTGCTGTGCTTACTGTTGTTGCT GCTCCCCCAACTCCAGCTATCATT 1785
596 Y A R P N P P L D L E L T G Q L E R S I 615
1786 TACGCT⇀CGGCCAAATCCACCGCTTGACTTGGAATTGACAGGTCAGCTAGAAAGAAGCATT 1845
■
616 E L S W V P G E E N N S P I T N F V I E 635
1846 GAACTCTCATGGGTACCAGGAGAAGAAAATAACAGTCCCATTACAAACTTTGTGATTGAG 1905
■ FNIII#1
636 Y E D G L H E P G V W H Y Q T E V P G S 655
1906 TATGAAGATGGACTACATGAGCCAGGGGTATGGCATTACCAGACGGAAGTTCCTGGATCT 1965
+ ■
656 Q T T V Q L K L S P Y V N Y S F R V I A 675
1966 CAGACAACTGTACAGTTGAAGTTGTCTCCGTATGTCAACTACTCATTCCGTGTGATTGCT 2025
676 V N E I G R S Q P S E P S E Q Y L T K S 695
2026 GTCAATGAAATTGGTAGAAGTCAGCCAAGTGAACCATCTGAACAGTACCTGACAAAGTCC 2085
696 A N P D E N P S N V Q G I G S E P D N L 715
2086 GCAAACCCCGATGAAAATCCTTCTAATGTACAAGGGATAGGCTCGGAACCTGATAATTTG 2145
■ ■
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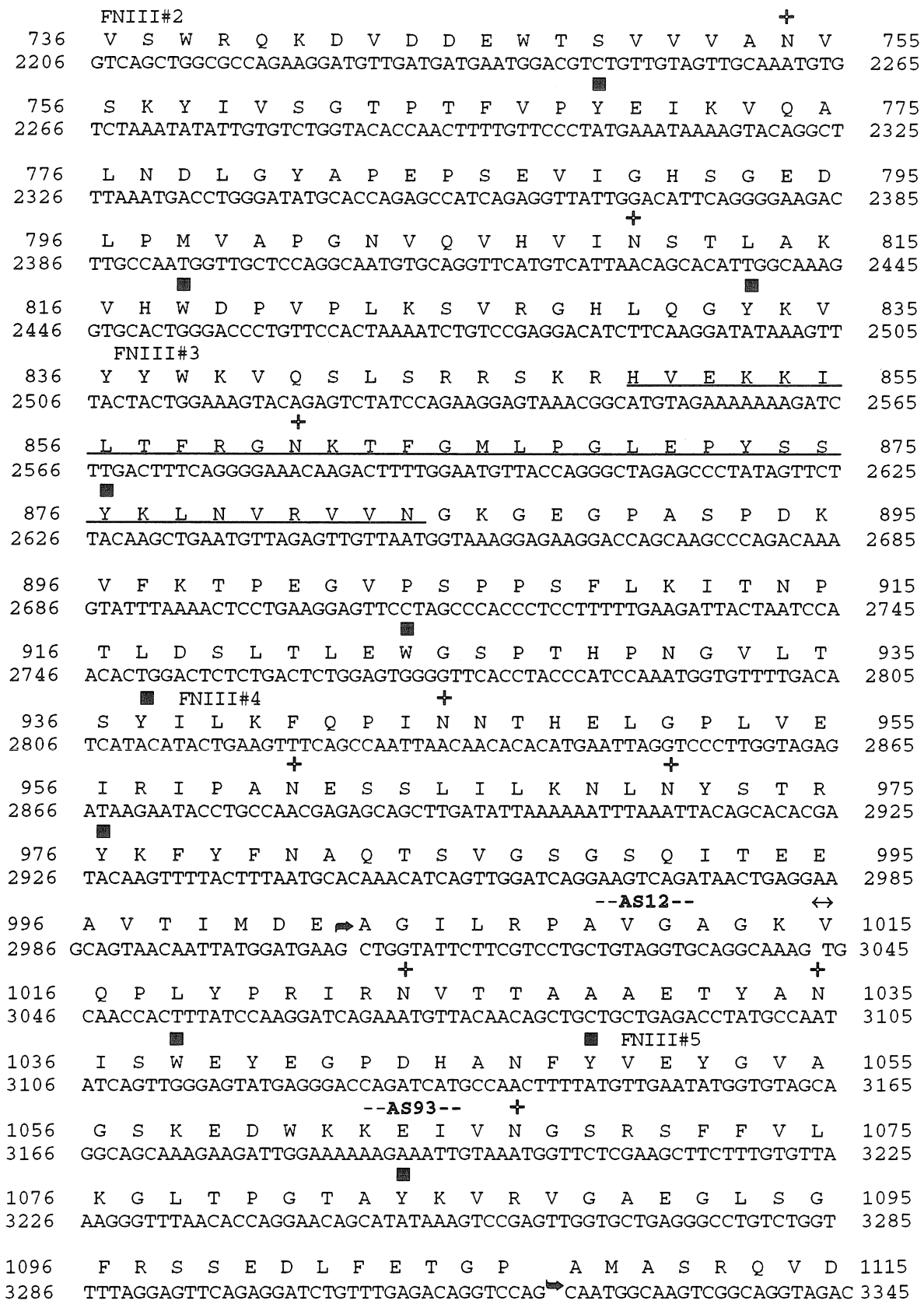
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756 S K Y I V S G T P T F V P Y E I K V Q A 775
2266 TCTAAATATATTGTGTCTGGTACACCAACTTTTGTTCCCTATGAAATAAAAGTACAGGCT 2325
776 L N D L G Y A P E P S E V I G H S G E D 795
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796 L P M V A P G N V Q V H V I N S T L A K 815
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2926 TACAAGTTTTACTTTAATGCACAAACATCAGTTGGATCAGGAAGTCAGATAACTGAGGAA 2985
--AS12--
996 A V T I M D E A G I L R P A V G A G K V 1015
2986 GCAGTAACAATTATGGATGAAG CTGGTATTCTTCGTCCTGCTGTAGGTGCAGGCAAAG TG 3045
1016 Q P L Y P R I R N V T T A A A E T Y A N 1035
3046 CAACCACTTTATCCAAGGATCAGAAATGTTACAACAGCTGCTGCTGAGACCTATGCCAAT 3105
FNIII#5
1036 I S W E Y E G P D H A N F Y V E Y G V A 1055
3106 ATCAGTTGGGAGTATGAGGGACCAGATCATGCCAACTTTTATGTTGAATATGGTGTAGCA 3165
--AS93--
1056 G S K E D W K K E I V N G S R S F F V L 1075
3166 GGCAGCAAAGAAGATTGGAAAAAAGAAATTGTAAATGGTTCTCGAAGCTTCTTTGTGTTA 3225
1076 K G L T P G T A Y K V R V G A E G L S G 1095
3226 AAGGGTTTAACACCAGGAACAGCATATAAAGTCCGAGTTGGTGCTGAGGGCCTGTCTGGT 3285
1096 F R S S E D L F E T G P A M A S R Q V D 1115
3286 TTTAGGAGTTCAGAGGATCTGTTTGAGACAGGTCCAG CAATGGCAAGTCGGCAGGTAGAC 3345

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1116   I  A  T  Q  G  W  F  I  G  L  M  C  A  V  A  L  L  I  L  I   1135
3346  ATTGCTACTCAAGGATGGTTCATTGGACTTATGTGTGCTGTTGCACTTCTTATCTTGATT  3405

1136   L  L  I  V  C  F  I  R  R  N  K  G  G  K  Y  P  V  K  E  K   1155
3406  TTACTGATTGTTTGCTTCATAAGGAGGAATAAAGGTGGCAAATATCCAGTGAAGGAAAAG  3465
                                                        *
1156   E  D  A  H  A  D  P  E  I  Q  P  M  K  E  D  D  G  T  F  G   1175
3466  GAGGATGCACATGCTGATCCAGAAATACAGCCTATGAAGGAAGATGATGGAACATTTGGT  3525
            *                                    *     *     *
1176   E  Y  S  D  A  E  D  H  K  P  L  K  K  G  S  R  T  P  S  D  R 1196
3526 GAATACAGTGATGCAGAGGACCATAAACCTCTAAAAAAAGGAAGTCGGACACCGTCAGACAGA 3588
       *                 *
1197   T  V  K  K  E  D  S  D  D  S  L  V  D  Y  G  E  G  V  N  G   1216
3589  ACTGTGAAAAAAGAAGACAGTGATGATAGTTTAGTTGACTATGGAGAAGGTGTAAATGGC  3648
                                          *
1217   Q  F  N  E  D  G  S  F  I  G  Q  Y  S  G  K  K  E  K  E  P   1236
3649  CAGTTCAATGAGGATGGCTCCTTTATTGGACAATACAGCGGTAAAAAAGAGAAAGAACCT  3708

1237   A  E  G  N  E  S  S  E  A  P  S  P  V  N  A  M  N  S  F  V   1256
3709  GCAGAAGGAAATGAAAGTTCTGAGGCTCCTTCTCCTGTAAATGCCATGAATTCATTTGTG  3768

3769  TAATCAAAGAACTTGATTCCCTTGTGTTTTCTGTTTGTTTGCACTTGTACATCCTCCTTC  3828
3829  TCGTACGATGAACATGCAGGTTACAAAGCTCCTCACCTCAAAGTATTTATTCTATGAGAA  3888
3889  CGTGCCGGTCCATACAATTATATGGTATAATGACACACCAAG                    3930
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The initiation codon, signal peptide and first 35 amino acids of the mature protein are missing from these data. The immunoglobulin-like domains are numbered Ig I through Ig VI over the conserved tryptophans (W) with the characteristic cysteines (C) denoted by circles numbered 1-6. The fibronectin type III repeats are numbered FNIII#1 through FNIII#5 with conserved tyrosines (Y) and tryptophans indicated by closed squares. Nucleotides encoding the transmembrane region are underlined. Underlined amino acids denote sequences determined by peptide sequence analyses of the 80 kD form of Bravo and of V8 proteolytic fragments of Bravo 130/140. Potential sites of asparagine-linked glycosylation are marked with (+), and potential phosphorylation sites are indicated by (*). Arrows separate putative alternatively spliced exons labeled AS10, AS12 and AS93. The putative splice site between adjoining sequences AS12 and AS93 is denoted by two-way arrow. The sequences of alternatively spliced cytoplasmic sequences, AS-CYT1 and AS-CYT2, are omited from this figure and can be found in Chapter IV, Fig. 6.