Molecular Characterization of a Receptor for the Togavirus Sindbis Virus

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Techology

Pasadena, California

1991

(Submitted May 22, 1991)

Acknowledgements

During my graduate study at Caltech, I have grown a lot personally as well as scientifically. I would like to express my first thanks to James and Ellen Strauss for their constant consideration and encouragement. I am especially grateful for his supervision policy of letting me be my own and solely responsible for directing my research, which contributed toward my a great progress in scientific growth. I also would like to thank my thesis committee members, Drs. Norman Davidson, Paul Patterson, Scott Emr, and Ellen Rothenberg for helpful suggestions in my thesis work.

I have learned a lot of different things from a number of people in Caltech, especially Richard Kuhn who has also been like a second advisor to me, with many advices about science and life. I am also grateful to Susan Ou for her collaborations and enthusiasm to generate useful monoclonal antibodies to keep the project running. Thanks to Rochelle Diamond for her excellent technical assistance in flow cytometric analysis, to Louis Gastinel and Wen-Ching Wang for help in transfection assay. Many thanks to Frank, Yukio, Till, Raoul, Nathalie, Hong, Chen-Wen and Edith for your scientific suggestions and friendship.

Finally to my mother, who with limited economic resources , supported me and my two young brothers, making it possible for us to complete our higher education during last twenty years. Without her encouragement, many facts will never be come truth in my life.

Abstract

The first step in any virus entry process is binding to the plasma membrane of the host cell. The nature of this obligatory step depends upon both the viral and cellular components and may be quite divserse among viruses. The entry of Sindbis virus into a host cell is reported to occur via receptor-mediated endocytosis. We have used several approaches to isolate and characterize the receptor(s) for Sindbis virus.

In one such approach, we searched for monoclonal antibodies (mAb) that could interfere with Sindbis virus attachment to and infection of baby hamster kidney (BHK) cells. Mice were immunized with multiple injections of whole BHK cells or with BHK cell membranes. Hybridomas were prepared and supernatants from approximately 3600 hybridoma clones were screened by a plaque reduction assay for their ability to interfere with virus infection. One IgM mAb from a mouse immunized with whole BHK cells inhibited Sindbis virus attachment to BHK cells by 80% at 20 μg/ml, and immunoprecipitated a 68 kDal membrane protein from BHK cells. This mAb also inhibits virus attachment to two other mammalian cell lines tested, Vero cells (monkey) and SW13 cells (human), and also immunoprecipitates a 68 Kd protein from these cells. The mAb does not interfere with virus infection of chicken cells but did immunoprecipitate a 71 kDal protein from chicken cells. This mAb was used to screen 10^6 plaques from a λgt11 cDNA library from BHK cells, and 15 reactive phages were found. Six of the fifteen were shown by sequence analysis to react with overlapping regions of a protein that was identical in sequence to the mouse high affinity laminin receptor. By rescreening with a probe from one of these reactive phages, other lambda phages containing the remaining regions of the gene were found. The complete sequence of this protein was deduced by sequence analysis of the cDNA clones and was identical to that of the mouse laminin receptor and 99% identical to the human laminin receptor. A full length cDNA clone of the gene was constructed and inserted into a high efficiency expression vector. BHK cell lines stably transfected with vector expressing the plus sense BHK laminin receptor cDNA

are 3-5 fold more susceptible to infection by Sindbis virus as measured by plaque assay, and overexpress the receptor protein on their surface as assayed by flow cytometry analysis. Conversely, cell lines transfected with vector expressing antisense laminin receptor cDNA are only about one half as susceptible to infection by Sindbis virus as the nontransformed BHK cells, and expression of laminin receptor on the cell surface is reduced as measued by flow cytometry analysis.

In a second study we looked for Sindbis virus receptors on the surface of chicken cells, using specific molecular mimicry to identify receptor molecules. It has been postulated that viral receptors may share structural features (idiotypes) with antibodies directed against the cell attachment protein of virus. Using antiidiotypic antibodies directed against Sindbis-specific neutralization antibodies, we have demonstrated that an antiidiotypic antibody to a neutralizing mAb reactive with the E2 glycoprotein of Sindbis virus specifically interferes with the binding of wild type Sindbis virus to chicken cells. This antiidiotypic antibody also immunoprecipitates a 63 kDal protein from chicken cells and binds to the surface of these cells. This 63 kDal protein is presumably a receptor for Sindbis virus in chicken cells. The relationship between this protein and the laminin receptor used as a Sindbis receptor in mammalian cells remains to be determined.

We also wished to determine the domains of the virus envelope proteins that are responsible for attachment to the cell membrane. The Sindbis virus envelope contains two species of integral membrane glycoproteins, E1 and E2, which assemble into heterodimers. Each spike on the surface of the virion is a trimer of these dimeric units. We attempted to map the neutralization epitopes on the surface of the virus, including epitopes implicated in virus binding to cells by the antiidiotypic antibody results described above. A λgt11 expression library was constructed containing cDNA inserts 100-300 nucleotides in length obtained by randomly primed synthesis on Sindbis genomic RNA. This library was probed with several neutralizing monoclonal antibodies specific for E2 and one neutralizing antibody specific for E1. Four positive clones, all of which

contained inserts from the region of the Sindbis genome that encodes amino acids 173 to 220 of glycoprotein E2, were found from the screening with mAb 23. No reactive clones could be identified using any of the other antibodies. We hypothesize that this domain of E2 centered at residue 200 forms part of the virus binding site for attachment to the cell to initiate infection.

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Chapter 1

Introduction

Entry of Alphaviruses into Their Host Cells

A. The importance of studying virus entry

Living organisms of microscopic size include bacteria, fungi, protozoa and the agents at the borderline of the life, i.e. the viruses (Fraenkel-Conrat and Kimball, 1982; Davis et al., 1973). They have significant effects on human beings and on other animals and plants (Pelczar et al., 1986b), and even make physical and chemical changes to our environment (Pelczar et al., 1986a). In particular, detrimental microorganisms which cause human illness, or other animal and plant diseases, can result in great economic losses as well as losses in human health and welfare. With the invention of the light microscope and other research tools to help us to explore the world of microorganism, the study in the form, structure, reproduction, physiology, metabolism and classification of microbial agents has became feasible (Norris, 1990). Many antibiotics and other chemicals such as chloroquine have been developed to treat human diseases caused by many bacteria, fungi and protozoa (Gottlieb and Shaw, 1967; Weinstein, 1970; Agabian and Cerami, 1989) and today many humans are treated and recover from infectious diseases such as leprosy and malaria, which were prevalent during medieval times, often with devastating mortality (Ciba, 1963).

Viruses, on the other hand, cause many major human infectious diseases prevalent today. Due to their small size they cannot be observed directly under the light microscope; since they are obligate intracellular parasites, they cannot be cultured (like bacteria) on artificial laboratory media. Consequently, the identification of viral pathogens is more difficult than identification of bacteria pathogens. During the last fifty years, cell tissue culture systems (Enders et al., 1949) and electron microscopy techniques (Ruska, 1940) have been developed to provide a means by which many viral pathogens can be cultivated and observed in the laboratory, although certain human pathogens, such as the Norwalk agent, have never been successfully grown in culture. In addition in this decade recombinant DNA techniques and immunological methods have played major roles in

fundamental research on the nature of viruses, on their replication and maturation in their hosts, and on animal immune responses to virus infection (Maramorosch and Koprowski, 1984; Quinnan, 1984). Although virology has become a better established field among the life sciences, in many cases our current knowledge is insufficient to prevent viral infection effectively. With the exception of several safer and less expensive recombinant DNA vaccines (such as the hepatitis B vaccine) (Ginsberg et al., 1988) (Purcell and Gerin, 1987) and a few modestly effective antiviral drugs (such as interferon (Baron, 1966)) and the nucleotide analogs like AZT (Yarchoan et al., 1988; Chaisson and Allain, 1986), viral disease can only be prevented by traditional epidemiological methods such as quarantine to prevent diagnosed ill individuals from transmitting viruses to healthy persons and immunological methods such as vaccination. Therefore, the search for the additional effective methods of preventing viral infection has become a major target for virologists.

For last 30 years, many virologists have tried to answer the question, "How does a virus enter a cell and deliver its message into the cytoplasm?" Except for a few well-studied cases, this is still the biggest "black box" in virus research, and closely related to studies on the nature of pathogenicity. Viruses that can cause human disease may be strictly limited to human hosts such as Epstein-Barr virus (Henle and Henle, 1979), or they may be mainly pathogens of humans, with other animals serving only as accidental hosts, such as influenza virus (Fox and Kilbourne, 1973; Stuart-Harris et al., 1976). Many viruses have very specific tissue tropisms (such as hepatitis B which infects exclusively human liver (Juretic, 1980; Nahmias et al., 1981), but some viruses have weaker tissue tropisms such as yellow fever, which infects a variety of distinct tissues in addition to the liver (Strode, 1951; Theiler and Downs, 1973).

In the case of viruses with very specific host or tissue tropisms, certain essential components may be present in or on infectible cells, which do not exist in nonsusceptible cells. Similarly, viruses with weak tissue tropisms may use common cellular components present on many types of cells, or a similar biological uptake process for viral infection.

Thus there seem to be a number of virus-host cell interactions which determine pathogenicity and distinguish susceptible from nonsusceptible cells, as outlined below:

(1) It has been hypothesized that infectible cells contain specific factors which are involved in virus replication and assembly such as polymerases, capping enzymes, and proteases, which are therefore essential for virus maturation (Fenger, 1984; Weiss et al., 1982). Similarly, uninfectible cells in the same host or cells from nonsusceptible hosts have been assumed to lack these necessary factors. In a number of well-studied cases, however, it has not been possible to distinguish between susceptible and nonsusceptible cells on the basis of enzyme activities thought to be essential for viral replication, in part because host enzymes as such as polymerases are not only involved in viral replication, but also support the production of host RNAs. Moreover, many of these factors are essential to maintain cellular metabolism, and independent of the state of differentiation of the host.

(2) Another hypothesis is that biological uptake processes such as pinocytosis or phagocytosis play a major role in virus uptake, similar to the uptake of toxins or antigen-antibody complexes. However, it is unclear why particular unsusceptible cells should fail to take in viruses, since pinocytosis and phagocytosis are common biological phenomena (Steinmen et al., 1983; Pastan and Willingham, 1983; Griffin et al., 1975).

(3) Finally, there has been speculation that cells infectible with a particular virus have certain specific molecules in common on their surfaces which are recognized by that virus and that the existence of specific common components on infectible cells may be due to differences in differentiation within distinct tissues. Examples of specific proteins produced in distinct differentiated cells are cellular protein growth hormones produced in the brain and insulin generated in the pancreas (Pecile and Muller, 1968). Identification of cellular receptors for viruses will help us to understand how viruses enter cells and how they cause disease.

Based on the well-understood animal immune responses to virus infection (Quinnan, 1984), vaccines and diagnostic methods have been developed as the most

successful strategy to prevent viral infection (Menegus, 1984; Maramorosch and Koprowski, 1984), but vaccination still has many shortcomings. For example, vaccination must be administered before the host is exposed to the virus; the host immune response to a vaccine may not be consistant in all individuals; antibody titer in response to vaccination may decrease with time; a vaccine to one serotype of a virus with multiple serotypes may not protect against the prevalent serotype in a host population (for example, there are >100 serotypes of rhinovirus which cause the common cold); and certain viruses like HIV are inserted into host chromosome DNA and escape vaccine triggered antibody interaction (Ginsberg, et al., 1988; Haseltine et al., 1989).

Recently, as virus replication becomes better characterized on a molecular level, chemotherapeutic antiviral drugs have been developed which appear promising for treatment of infected patients. Examples of agents which have been used to treat patients include nucleotide analogs like AZT, which blocks HIV DNA synthesis, and several viral protease inhibitors which prevent cleavage of viral protein precursors. However, problems in delivery of such drugs into infected cells and toxic side effects have reduced their role in preventing virus infection (Van Voris, 1984).

Research on the possible surface components of infectible cells which interact with viruses will provide important knowledge not only on viral pathogenicity but help in the future design of antiviral drugs which can prevent viral infection by preventing the initial step, virus attachment to cells. At the current time many promising techniques to prevent virus entry, such as specific anti-receptor antibodies, high affinity anti-receptor inhibitor and chemical synthesized receptor compound to trap viruses are being developed.

B. Possible mechanisms of virus entry

During the past 20 years the pathways of viral entry into cells have been investigated by electron microscopy and cell biological techniques in which viruses can be visualized bound to the cell surface prior to entry and within cells after entry. Some

investigators have shown that some viruses enter the cell in a vacuole or vesicle which was induced by the presence of the virus at the cell surface. The uptake process for such viruses, which are recognized as opportunistic ligands like toxins, hormones, and growth factors, is a receptor-mediated form of endocytosis, i.e., constitutive pinocytosis (Steinmen, et al., 1983; Pastan and Willingham, 1983; Silverstein et al., 1977). During receptor-mediated endocytosis ligands first bind to cell surface receptors to form ligand-receptor complexes, after which the region of the plasma membrane containing the ligand-receptor complexes is internalized by endocytosis at clathrin-coated pits, which are specialized depressions on the cell surface. Coated pits containing ligand-receptor complexes fold inward and pinch off into the cytoplasm to form "coated vesicles." These endocytic vesicles usually have one of three possible fates. 1) The vesicle can be transported to the other side of the cell and exocytose its inner contents, 2) the vesicle can fuse with other vesicles to store its contents within the cell, or 3) in most cases the endocytic vesicle fuses with other cellular organelles such as endosomes (Helenius et al., 1980) to form an intermediate transport vesicle with subsequent degradation of contents and recycling of membrane components (Davey et al., 1985). Electron microscope images published by Dales and co-workers 20 years ago show adenovirus entering cells via coated pits and endocytic vesicles in the same pathway as epidermal growth factors (EGF) (Dales, 1962).

Other investigators have demonstrated that viruses can enter cells directly across the plasma membrane without being transported in vesicles. In other words, entry might occur by fusion of the viral membrane with the membrane of the cell. Images have been obtained in the transmission electron microscope which show the two opposing cytoplasmic leaflets of viral membrane and plasma membrane merging into one with a trilaminar structure replacing the previous tetralaminar one. The point of contact between the two membranes widens slightly for fusion processing allowing the hydrophobic regions of the extracytoplasmic leaflets of the two membranes to contact one another. Finally, the two

extracytoplasmic leaflets fuse and the membrane fusion event is completed (Wilschut and Hoekstra, 1983; Darnell et al., 1986) with concommitant release of the viral nucleocapsid into the cytoplasm for replication. Such fusion has been observed among enveloped viruses such as Sendai virus and influenza A virus.

C. Entry of Alphaviruses

Among those viruses which have been shown to enter cells via coated pits and endocytic vesicles are enveloped viruses such as Semliki Forest virus and vesicular stomatitis virus (White et al., 1981). Semliki Forest virus and the closely related Sindbis virus are the most well-studied members in the alphavirus group of the Togavirus family. For the past 15 years the laboratory of Helenius has been studying Semliki Forest virus entry in detail. The evidence mainly from electron microscopy shows that alphaviruses enter cells by receptor-mediated endocytosis (Garoff et al., 1982; Helenius, et al., 1980). The first stage in entry is the attachment of the viral spike glycoproteins to the cell surface, and the virus particle binds to the surface components (receptors) of the plasma membrane. After binding to a surface component the virus particle moves toward the center of the cell. Within a few minutes this motion takes the particle into a "coated pit," which pinches off into the cytoplasm, forming an endocytic "coated vesicle." Within a short time the coated vesicle has moved into cytoplasm and fused with an endosome, which provides an acidic environment (pH 5.0-6.0) for alphavirus penetration (Helenius et al., 1983). A virus particle spends about 60 min in the endosome compartment on average until the endosome fuses with a lysosome, an organelle which degrades unneeded substances (Marsh et al., 1983). The acidity of the interior of the lysosome has been reported to induce a change in the viral membrane enabling it to fuse with the lysosomal membrane. The fusion is so rapid that the viral nucleocapsid is expelled into the cytoplasm before it can be destroyed by the degradation enzymes of the lysosome (Helenius, et al., 1980; Marsh and Helenius, 1980). In the cytoplasm, the genomic RNA is released from the capsid proteins to function

as messenger RNA (Helenius et al., 1982) for the nonstructural proteins, which are components of the viral RNA-dependent RNA polymerase.

D. Identification of cell surface receptors

Several approaches which will be discussed below have been used to identify cell surface receptors for viruses.

1. Analysis of virus receptors by hemagglutination assay

Since the initial event in the interaction of a virus with the host cell is the attachment of the virus to receptors in the plasma membrane, the identification of cell surface receptors is crucial for understanding the early stages in the interaction between virus and host cell. Many investigators have estimated that there are only about 10^4 to 10^5 receptors per host cell, making it difficult to obtain sufficient material for traditional biochemical characterization (Smith and Tignor, 1980; Fries and Helenius, 1979). However, one feasible approach to the analysis of the cell surface components that mediate viral attachment to erythrocytes is a sensitive hemagglutination assay. Some virus products can cause agglutination of red blood cells; this phenomenon is named hemagglutination (Howe and Lee, 1972). The analysis of hemagglutinating activity was used in immunological tests to characterize the surface components of virions and the virus adsorption to the cells (Bachi et al., 1977). By this indirect means several erythrocyte components were identified as viral receptor sites. Glycophorin A, the major glycoprotein of the erythrocyte membrane, containing nearly 90% of the total cell-surface sialic acid (Bachi et al., 1977), has been purified to test its ability to bind to animal viruses. Purified human glycophorin A inhibits hemagglutination by influenza virus (Marchesi and Andrews, 1971; Enegren and Burness, 1977) encephalomyocarditis (EMC) virus (Enegren and Burness, 1977) and small-plaque polyomavirus (Cahan et al., 1983), and purified bovine glycophorin A also inhibits hemagglutination by Sendai virus and Newcastle disease virus (Suzuki et al., 1983). This means that glycophorin A can bind specifically to

these viruses to prevent hemagglutination. The other major erythrocyte membrane protein, Band 3, containing two sialic acid residues, has been shown to interact with Sendai virus to cause aggregation to form particles. This result suggested a direct interaction between Sendai virus with Band 3 (Nigg et al., 1980). The haemagglutination assay only gave a limited contribution to the identification of possible virus receptors on the erythrocyte surface; the relationship between receptors on erythrocytes leading to agglutination and receptors on susceptible host cells leading to infection in unclear.

2. Isolation of viral high-affinity receptors

Another approach to the isolation and characterization of cell surface receptors has been based on the assumption that receptors on the cells will bind the viral attachment protein with high affinity. Various investigators have attempted to isolate putative receptor proteins from detergent-solubilized host cell membranes by affinity chromatography on an insoluble matrix containing covalently bound viral attachment protein. To identify the adenovirus type 2 receptor, Svensson et al. (1981) analyzed Triton-X-100 solubilized HeLa cell membranes on an affinity matrix consisting of adenovirus type 2 virions bound to Sepharose and isolated a 40kDa to 42kDa protein which bound to the column with high affinity (Nigg, et al., 1980). Using a similar matrix, Hennache (1977) found additional virus receptor candidates with molecular weights of 78 kDa and 34 kDa as well as 42 kDa protein described above from solubilized KB cell membranes (Hennache and Boulanger, 1977). On the other hand, Meager et al. (1976) reported that a 100 kDa glycoprotein from KB cells bound with high affinity to a column of adenovirus type 5 fibers coupled to Sepharose (Meager et al., 1976).

Helenius et al. (1978) has proposed that human (HLA) and murine (H2) histocompatibility antigens may serve as cell surface receptors for Semliki Forest virus based on the fact that histocompatibility antigens were the major proteins isolated by an Semliki Forest virus spike protein affinity column and were also the predominant cell proteins isolated by immunoprecipitation of soluble spike protein-membrane complexes

with spike protein antibodies (Helenius et al., 1978). However, other membrane components in addition to histocompatibility antigens must serve as cell surface receptors for Semliki Forest virus, since murine F9 and pcC4 teratocarcinoma cells which do not contain H2 histocompatibility antigens are infectible by Semliki Forest virus (Oldstone et al., 1980).

Ambiguous results have also been reported in the identification of the C3d receptor for complement as the Epstein-Barr virus receptor (Jonsson et al., 1982; Simmons et al., 1983) and in the identification of a protein of 140 kDa as the receptor for Friend murine leukemia virus (Robinson et al., 1980; Bubber et al., 1978). Various laboratories have used similar approaches to identify these virus receptors, but the results were not completely consistent and there appear to be major technical difficulties with the affinity columns. Several possible explanations for the inconsistent results include the fact that the whole virions are frequently degraded during the coupling to the matrix, virion proteins coupled to columns are not recognized by the solubilized receptor protein in the same way as virus particles are, and the presumptive receptor proteins in the detergent-treated membrane preparations may not maintain a sufficiently native conformation to interact with the virus.

3. Purification of viral receptors by anti-idiotypic antibodies

Based on Jerne's internal image hypothesis it should be possible to isolate a virus receptor using as an antireceptor antibody an anti-idiotypic antibody to the paratope of an anti-virus attachment protein antibody. Theoretically it should be easy to purify the receptor, using the antiidiotypic antibody, assuming that the antiidiotype contains a similar structure for attachment to receptor as the virus (a "pseudovirus") but with higher affinity for binding to the receptor than the virus (Farid and Lo, 1985a). This approach has been used successfully to isolate the receptor for reovirus type 3; polyclonal anti-idiotypic antibodies to a mouse monoclonal anti-virion sigma 1 antigen effectively bound to the 67 kDa cellular receptor (Co et al., 1985b; Co et al., 1985a). When the isolated receptor was

blotted on nitrocellulose paper it bound not only the antiidiotypic antibody but also virus. Although antiidiotypic antibodies seem to be feasible tools for receptor isolation, they been used successfully in only a limited number of cases such as the identification of the TSH receptor (Farid and Lo, 1985b), the insulin receptor (Shechter et al., 1982), the CD4 receptor for HIV (Dalgleish et al., 1987) and the 67Kd receptor for reovirus (Co et al., 1985b). This approach is not generally applicable, because the antigenic site of the original antibody may be embedded in the interior of the heavy chain and light chain complex and its immunogenicity can be poor such that few anti-idiotypic antibodies are produced.

4. Identification of anti-receptor antibodies

A more generally applicable strategy uses anti-receptor antibodies for receptor purification. Monoclonal antibodies to receptors from hybridomas from mice immunized with host whole cells or cell plasma membranes may be screened for their ability to protect cells against virus attachment and infection. In this way monoclonal anti-receptor antibodies have been successfully selected which recognize the receptors for human rhinovirus (Colonno et al., 1986), poliovirus (Nobis et al., 1985), and Group B coxsackieviruses (Campbell and Cords, 1983). However, monoclonal anti-receptor antibodies are usually generated at a very low frequency, with only one in several thousand hybridoma clones specific for the virus receptor. Even if the mAb prevents virus infection, the sites of virus attachment to the receptor may not be identical or proximal to the epitopes recognized by monoclonal anti-receptor antibodies. Due to the difficulties in screening large numbers of monoclonal antibodies for their ability to prevent virus infection, only a few anti-receptor antibodies have been identified by screening libraries of anti-cell antibodies for virus blocking activity.

E. Characteristics of known virus receptors

Progress in the analysis of cell surface receptor on host cells was hampered for many years by the lack of suitable assays for receptor activity. However, recently several cases have shown success. The best-defined receptors are those for two enveloped viruses, influenza viruses (Weis et al., 1988) and human immunodeficiency virus (HIV) (Klatzmann et al., 1984; Maddon et al., 1986), and two nonenveloped viruses, poliovirus (Mendelsohn et al., 1989) and rhinovirus (common cold virus) (Greve et al., 1989).

Previous work had shown that the erythrocyte glycoprotein, glycophorin A, might be related to influenza virus attachment (Enegren and Burness, 1977; Marchesi and Andrews, 1971). The fact that influenza virus might bind to sialic acid was first suggested by showing that treatment of cells with neuraminidase destroys their capacity to bind virus and that reintroduction of sialic acid restores the capacity to bind virus. Further evidence that the receptor contains sialic acid was obtained by demonstrating that carbohydrates containing terminal sialic acid residues compete for virus binding. Weis et al. have now determined the three-dimensional structure of the influenza hemaglutinin, HA, complexed with sialyllactose, a trisaccharide composed of sialic acid, galactose, and glucose and shown that sialic acid is influenza virus receptor (Weis et al., 1988).

The best-characterized proteinaceous viral receptor is the CD4 glycoprotein, the HIV receptor. Early studies had demonstrated that *in vitro* infection of $CD4^+$ cells could be blocked by anti-CD4 antibody (Klatzmann et al., 1984), and that the viral envelope glycoprotein, gp120, remained bound with high affinity to CD4 after solubilization of cell-bound viral particles. Confirmation that CD4 was the functional HIV receptor was obtained by demonstrating that previously uninfectable human brain cells could bind virus and become infected after they were transfected with a CD4 cDNA (Maddon et al., 1986). Two other well-characterized proteinaceous viral receptors are the receptors for polioviruses and rhinoviruses; these viruses belong to two closely related genera in the family *Picornaviridae*. Both contain a single molecule of positive-strand RNA coated with

a proteinaceous icosahedral shell. Polioviruses infect humans causing paralytic poliomyelitis (Moore and Morens, 1984) and rhinoviruses cause the common cold (Gwaltney, 1982). An anti-receptor monoclonal antibody which blocks infection of a majority of human rhinovirus serotypes was found to react with a 90 Kd protein on HeLa cells (Colonno et al., 1986). This mAb was used to purify the sufficient amounts of the receptor to determine its amino acid sequence. From the sequence it was clear that the receptor is the cell adhesion molecule ICAM-1. Moreover, mouse cells, which are normally uninfectible by rhinovirus, became susceptible to virus infection after transfection with the ICAM-1 gene. This finding is also supported by the evidence that purified ICAM-1 specifically binds to the major rhinovirus serotypes (Greve et al., 1989).

The poliovirus receptor gene was isolated by transfecting human DNA into virus uninfectable mouse L cells, and selecting transfectants that could bind a monoclonal anti-receptor antibody that prevents virus infection. The sequence of the receptor cDNA analyzed from these transfectants, which are infectable with poliovirus, revealed that the poliovirus receptor, like the rhinovirus receptor and the HIV receptor, is a member of the immunoglobulin superfamily (Mendelsohn et al., 1989).

F. Sindbis virus infection

There are over 26 members in the alphavirus genus of the family Togaviridae, many of which are pathogenic for man and domestic animals. Many of the New World viruses such as Eastern, Western and Venezuelan equine encephalitis viruses cause neurological disease and encephalitis whereas the Old World viruses such as Sindbis and Semliki Forest viruses usually lead to fever, rash and arthralgia (Griffin, 1986; Peters and Dalrymple, 1990). These simple enveloped viruses are transmitted in nature by blood-sucking arthropods, and infect a wide variety of vertebrate hosts including birds and mammals. In the laboratory alphaviruses can be grown in a wide variety of cultured cells (see below).

Both Sindbis virus and Semliki Forest virus have been well characterized, and have low virulence in man (Griffin, 1986). The characterization of the host cell receptor for Sindbis virus and the interaction between Sindbis virus enveloped spikes and its receptor will be discussed in my thesis work.

Sindbis virus can infect many commonly-used tissue culture cells such as human adrenal cortex SW13 cells, monkey kidney (Vero) cells, mouse neuroblastoma N18 cells, baby hamster kidney (BHK) cells, chicken fibroblast cells (Strauss et al., 1969) and mosquito larvae C6/36 cells (Chamberlain, 1980). We previously tested the ability of bluegill fish BF-2 cells, brown bullhead fish trunk BB cells, viper spleen VSW cells, *Xenopus* kidney A6 cells to be infected with Sindbis; Sindbis virus caused cytophathic effects in all of these cell lines after infection. Sindbis virus not only infects a wide variety of hosts from invertebrate mosquitos to vertebrate fishes, reptiles, amphibians, birds and mammals (Chamberlain, 1980; Niklasson, 1989), but it also shows weak tissue tropism in that the virus can infect cells from many distinct tissues such as kidney, neuroblastoma, fibroblast, spleen, trunk, larva, etc. This implies that the virus may recognize surface structures common to many different cell types. As previously described, influenza virus and Sendai virus utilize sialic acid as a receptor; both viruses have a wide range of hosts from human and bovine to chicken, and sialic acid also exists in many distinct cell types. But the initial hypothesis that sialic acid can function as a receptor for Sindbis virus does not seem to be true, since this sugar is not found in mosquito cells (Stollar et al., 1976) and neuraminidase treatment of host cells does not affect virus binding (Smith and Tignor, 1980). It is also possible that Sindbis virus uses different receptors in different cell types or in different hosts; similarly it may be true that different alphaviruses use different receptors. It is notable that among rhinoviruses, ICAM-1 is the receptor for 75 major serotypes, but that there are other receptors for other serotypes (Greve et al., 1989). If a conserved virus receptor exists in all cells infectable by Sindbis virus in its many distinct host species, it has been conserved during a long peroid of evolution.

If a specific receptor exists for Sindbis virus, the isolation of this receptor will be crucial for understanding interactions between the virus and its host. Since the initial interaction between virus and receptor may be characterized by low affinity, traditional ligand-coupling chromatography might not be a feasible method for purifying the Sindbis receptor. Therefore, we have attempted to identify and characterize the Sindbis-specific receptor on vertebrate tissue culture cells by 1) preparing monoclonal antibodies that block virus attachment to the receptor (Nobis et al., 1985; Minor et al., 1984; Campbell and Cords, 1983) and 2) using anti-idiotypic antibodies (to antiviral antibodies) which faithfully mimic the receptor-ligand interaction as specific receptor probes (Co et al., 1985b; Co et al., 1985a).

G. Characterization of Sindbis virus attachment sites

The structure, genome organization, and replication of Sindbis virus has been extensively studied at the molecular level. The mature virion contains a single 49S genomic RNA complexed with approximately 240 capsid protein subunits (MW ~ 30,000 daltons) to form an icosahedral nucleocapsid. The nucleocapsid is surrounded by a membrane consisting of a host-derived lipid bilayer in which are embedded two virus-encoded glycoproteins, E1 and E2 (each of MW ~50,000 daltons). These glycoproteins are found on the surface of the particle as trimeric associations of heterodimers which form spike-shaped protrusions. The spike proteins and viral membrane do not merely provide a protective coat for the nucleocapsid; they play a crucial role in the attachment of the virus to receptors on host cells. Analysis of the glycoproteins E1 and E2 showed a carboxyl-terminal hydrophobic region embedded in the bilipid layer, and that the ratio of these two glycoprotein is equimolar (Strauss and Strauss, 1986). Glycoprotein E1 contains the hemagglutination activity of the virus (Rice and Strauss, 1981; Dalrymple et al., 1976). Various antibodies specific for either E1 or E2 can neutralize, but glycoprotein E2 appears

to carry most of neutralization epitopes, which are candidates for possible virus attachment sites (Schmaljohn et al., 1983).

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Chapter 2

Molecular Characterization of a Receptor for the Togavirus Sindbis Virus

This chapter is being prepared for submission

Abstract

We show here that a high affinity laminin receptor serves as a receptor for Sindbis virus in mammalian cells. The primary amino acid sequence of this 295 residue laminin receptor is highly conserved in mammals, binds with high affinity to basement membrane laminin, and is known to be important in development and in tumor invasion and metastasis. The wide distribution of this conserved receptor may be responsible, at least in part, for the very broad host range exhibited by the virus.

Introduction

The initial event in the interaction of a virus with the host cell is the attachment of the virus to receptors in the plasma membrane (Lonberg-Holm and Philipson, 1974; Lonberg-Holm and Philipson, 1981; Tardieu et al., 1982). The presence of specific cellular receptors is to some extent a determinant of the species and tissue tropism of a virus and of its pathogenesis (Crowell and Landau, 1983; Holland, 1961; Sharpe and Fields, 1985) and characterization of such receptors is of considerable interest. Most animal viruses appear to use protein receptors, but carbohydrate receptors have been found in the case of two enveloped viruses, influenza virus and Sendai virus (Markwell et al., 1981; Weis et al., 1988). Protein receptors include CD4, expressed on the surface of lymphocytes and brain cells, that serves as receptor for human immunodeficiency virus (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986), the cellular adhesion molecule ICAM-I that serves as a receptor for human rhinoviruses (Greve et al., 1989; Staunton et al., 1989), an immunoglobulin-like protein that serves as a receptor for poliovirus (Mendelsohn et al., 1989), a β-adrenergic receptor that serves as a receptor for human reoviruses (Co et al., 1985a,b), and the C3d complement receptor CR2, expressed on human B lymphocytes, that serves as a receptor for the human herpesvirus Epstein-Barr virus (Fingeroth et al., 1984; Frade et al., 1985). In all of these cases of characterized

protein receptors, the virus exhibits a narrow host range both with respect to the animal host and to the tissues within the host that are infected.

There are 26 registered members of the alphavirus genus of the family Togaviridae, many of which are pathogenic for man and domestic animals, causing fever, arthralgia, or encephalitis (Griffin, 1986; Peters and Dalrymple, 1990). In nature, the viruses infect a wide variety of vertebrate hosts including birds and mammals and are transmitted by mosquitos, in which they also replicate (Chamberlain, 1980). Research has focused on Sindbis virus, the prototype alphavirus, and on Semliki Forest virus, both of which have a low virulence for man (Griffin, 1986). The mature Sindbis virion contains a single molecule of genomic RNA of 11703 nucleotides complexed with approximately 180 capsid protein subunits (MW ~30,000 daltons) to form an icosahedral nucleocapsid. This structure is surrounded by a lipid bilayer of host cell origin containing two virus specific transmembrane glycoproteins (E1 and E2) (each of MW ~50,000 daltons) organized in spike-like projections on the external surface of the virus particle (Strauss and Strauss, 1986). The spikes form the organs of attachment to host cell surface receptors. Experiments with Semliki Forest virus have shown that virions enter the cell through clathrin-coated pits and vesicles by receptor-mediated endocytosis. Following endocytosis, the virions are transported to endosomes where under acidic conditions the low pH triggers the fusion of the viral membrane with the endosomal membrane and penetration of virion RNA into the cytoplasms occurs (Helenius et al., 1980; Kielian and Helenius, 1986; Kielian et al., 1986; Marsh and Helenius, 1980; White et al., 1980).

We have previously reported on the use of antiidiotypic antibodies to study Sindbis virus receptors (Wang et al., 1991). We found that an antiidiotypic antibody made to a neutralizing antibody reactive with glycoprotein E2 appeared to function as an antireceptor antibody in chicken cells but not in BHK cells. In this paper we report the isolation of a monoclonal antibody that functions as an antireceptor antibody in mammalian cells. We have used this antibody to identify a mammalian receptor for Sindbis virus.

Results

A Monoclonal Antibody Directed Against BHK Cells Prevents Sindbis Virus Binding and Infection

Monoclonal antibodies (mAbs) which recognize the Sindbis virus receptor were raised using an approach similar to that used by others to identify viral receptors (Campbell and Cords, 1983; Minor et al., 1984; Nobis et al., 1985; Tomassini and Colonno, 1986). Hybridomas were prepared from 6 week old Balb/c female mice immunized at three week intervals with eight intraperitoneal injections of 10^7 whole BHK cells (two mice) or 200 μg sucrose gradient purified BHK membranes (one mouse) (Bringman et al., 1987), and ~3600 hybridoma culture supernatants were screened by plaque assay for their ability to protect BHK cell monolayers against infection by Sindbis virus strain AR339. One hybridoma from one of the mice immunized with whole BHK cells (out of ~1500 screened from this mouse) was obtained that secreted an IgM (mAb 36B12 1C3, hereafter referred to as 1C3) that partially blocked virus binding to BHK cells. An experiment illustrating the inhibition of plaque formation in BHK monolayers by 1C3 as a function of the concentration of purified mAb is shown in Fig. 1. In the presence of 20 μg/ml of 1C3, plaque formation was inhibited by 80%. Control mAbs, including 36B12 2B7 and 36C4 2G8 (both IgM) and 3H4 1B11 and 4F1 1H2 (both IgG_1), did not reduce the plaque titer.

In a second experiment, the ability of 1C3 to block the binding of saturating amounts of ^{35}S-labeled Sindbis virus to BHK cells was determined (Wang, et al., 1991). In the presence of 20 μg/ml mAb 1C3 only 28% as much Sindbis virus bound to BHK cells as was bound in the absence of competing mAb or in the presence of control mAbs (Table 1). Thus, mAb 1C3 blocked Sindbis virus binding to BHK cells as well as virus infection of these cells, and the plaque assay and virus blocking assay gave comparable results.

In order to demonstrate that 1C3 would bind to the surface of BHK cells, as would be predicted if it functions as an antireceptor antibody, we performed a flow cytometric analysis, and found that this mAb did bind to whole BHK cells (Fig2). We also considered the possibility that the mAb might block virus binding to cells by binding to the virus. An ELISA test using purified virus demonstrated no detectable binding of 1C3 to virus (data not shown).

Immunoprecipitation of a Sindbis Virus Receptor by 1C3

We first examined whether sialic acid might serve as a Sindbis virus receptor, as it does for influenza virus (Weis et al., 1988). In an ELISA, 1C3 did not bind to sialic acid, whereas a mouse anti-sialic acid mAb did bind (data not shown). We next examined whether mAb 1C3 would immunoprecipitate a protein from BHK cells. ^{35}S-labeled BHK cell membranes were prepared (Bringman et al., 1987) and proteins immunoprecipitated with 1C3 and various control mAbs, using a rat anti-mouse IgM-Sepharose (Zymed) as a second antibody (Hardy and Strauss, 1988; Wang et al., 1991; Young and Davis, 1983a). Analysis by SDS-polyacrylamide gel electrophoresis under reducing conditions revealed that a single labeled polypeptide with an apparent molecular mass of 68,000 daltons was precipitated by mAb 1C3, whereas no specific protein was immunoprecipitated by any of the control mAbs (Fig. 3).

Molecular Cloning and Sequencing of a Sindbis Virus Receptor

An oligo(dT)-primed cDNA library in λgt11 was prepared from BHK poly(A)$^+$ RNA and screened with mAb 1C3, using ^{125}I-goat-anti-mouse IgM (Dupont) as the secondary antibody (Young and Davis, 1983a,b). Fifteen reactive phage clones were found upon screening of 10^6 plaques. Of these, 6 clones designated λ7, λ11, λ15, λ26, λ30 and λ51 were found by Southern analysis to contain inserts that cross hybridized (Sambrook et al. 1989). Sequence analysis of these 6 inserts revealed that they all

contained the carboxy-terminal coding region of the hamster high affinity laminin receptor as shown by its identity to the mouse laminin receptor (Makrides et al., 1988; Rao et al., 1989) (Fig. 4). Thus it seems clear that mAb 1C3 is directed against the C-terminal domain of the high affinity laminin receptor. Four additional clones reactive with mAb 1C3 were also sequenced, and found to contain apparently irrelevant inserts: one insert encoded part of the ribosomal protein S26, one insert encoded a protein related to elongation factor EF2R, and two contained sequences not present in the protein data base.

In order to identify clones containing the 5' region of the laminin receptor gene, PCR primers were made using the known sequence from λ clone 25 and the lacZ fusion protein region in the λ vector, and a number of additional λ clones probed for the presence of long 5' regions. Several clones were identified that appeared to contain a complete 5' region, and 3 of these were sequenced. One of these, clone 3, is illustrated in Fig 4. The composite sequence obtained includes 64 nucleotides of the 5' nontranslated region, the entire 885 nucleotide sequence encoding the laminin receptor, and the 68 nucleotide 3' nontranslated region, and both chromosome copies appear to be represented (Fig. 5). The deduced amino acid sequence of the hamster laminin receptor is 295 residues in length and is identical to that of the mouse protein (Makrides et al., 1988), while differing at only 2 amino acids from the human protein (Wewer et al., 1986; Yow et al., 1988). The nucleotide sequence within the coding region is 92.5% identical between hamster and mouse, and 89% identical between hamster and human. A Northern analysis was performed to examine the size of RNA transcripts reactive with these cDNA clones. A 1.5Kb transcript in BHK cells and Vero cells was observed using low strigency wash, but no significant signals were seen in mosquito C6/36 cells or chicken fibroblast cells (Fig. 6). It suggested the nucleotide sequence of the BHK laminin receptor has a greater sequence identity with the monkey cell receptor than with the mosquito cell or chicken fibroblast cell receptor.

The 295 residue laminin receptor contains a putative short transmembrane domain at amino acids 86-101, and no signal peptide is predicted by the cDNA-derived sequence (Yow et al., 1988). There are two cysteine residues at positions 148 and 163, which most

likely form an intradisulfide bond in the middle of the molecule. The C-terminal domain from residue 160 to the carboxy-terminus is extracellular and contains the binding site for laminin. The terminal third of this region has a high negative charge, with 25% of the residues being aspartic acid or glutamic acid. Clones λ7, λ11, λ15, λ26, and λ30 are all immunoreactive with mAb 1C3 and all contain a 48 amino acid region in common, suggesting that the domain of the receptor recognized by the virus is within this C-terminal region.

Laminin and the synthetic peptide YIGSR, both of which bind to the laminin receptor (Cohen et al., 1989; Gehlsen et al., 1988; Graf et al., 1987; Yannariello-Brown et al., 1988), were examined for their ability to interfere with virus binding to cells. Neither of these polypeptides had any effct on virus binding, suggesting that the laminin receptor binding sites for laminin and for Sindbis virus are not spatially close.

Efficiency of Sindbis Virus Infection of BHK Cells that Overexpress the Laminin Receptor

Sindbis virus has a very broad host range, and attempts to identify cell lines completely lacking in receptors for the virus have not been successful. Thus it is not possible to transform a receptor negative cell to one that is susceptible to the virus, as has been done in other virus systems (Greve et al., 1989; Maddon et al., 1986; Mendelsohn et al., 1989). However, it seemed reasonable to expect that if the laminin receptor functions as a receptor for Sindbis virus, cells that expressed increased amounts of this receptor might become infectable with higher efficiency. For this purpose we constructed a full length cDNA clone of the BHK laminin receptor and inserted it in both the sense and antisense orientation into the expression vector pcDNA-1/neo (Invitrogen). This vector contains a kanamycin resistance gene and bacterial origin of replication for growth and selection in *E. coli*, and a neomycin resistance gene for selection of stable transfectants in mammalian cells. In this vector the inserted gene is expressed under the control of a cytomegalovirus promoter, a high efficiency mammalian promoter.

To construct the full length laminin receptor cDNA clone, inserts from clone 3 and clone 26 were excised from the lambda gt11 phages with *Eco*RI, and the purified inserts were digested with *Sac*I (there is a unique *Sac*I site in the laminin receptor sequence at position 564). After purification by agarose gel electrophoresis, the 5' end of the sequence from clone 3, from the *Eco*RI linker to the *Sac*I site, and the 3' end of the sequence from clone 26, from the *Sac*I site to the linker downstream of the poly(A), were joined in a three piece ligation with pcDNA/neo vector DNA which had been partially digested with *Eco*RI (since the *Eco*RI site in the polylinker is not unique). From this ligation, constructs containing the full length insert in both orientations were obtained, and plasmids LR pcDNA/neo-1 (sense) and LR pcDNA/neo-7 (antisense) were selected for transfection. BHK cells were transformed with both constructs as well as with the vector alone as a control, and neomycin resistant transformants were isolated. The efficiency of plaque formation was determined on multiple independent clones. Of 73 clones transfected with the plus sense gene, more than 90% demonstrated a greater than twofold increase in efficiency of plaquing of Sindbis virus, and 20% demonstrated a greater than fourfold increase in efficiency, as compared to vector only transformants (Table 2). Conversely, BHK cells transformed with the antisense gene demonstrated a decreased efficiency of plaquing (Table 2). This suggests that the laminin receptor gene is efficiently transcribed and expressed in transfected cells and that overexpression of the laminin receptor leads to increased susceptibility of BHK to infection by Sindbis virus, whereas expression of antisense RNA from the gene decreases the susceptibility of the cell, presumably by leading to decreased expression of the laminin receptor.

Four independent cell lines transfected with the plus sense gene (33, 40, 41, and 52) and three independent cell lines transfected with the antisense gene (4, 6, and 38), were selected for further study. The relative efficiency of plaque formation by Sindbis virus on these cells is shown in Table 3.

Expression of Laminin Receptor in BHK Transfectants

To confirm that BHK cells transfected with the laminin receptor gene in the plus orientation expressed increased amounts of laminin receptor on their surface, as expected from their increased susceptibility to infection by Sindbis virus, we assayed the ability of these cells to bind mAb 1C3 by fluorescence-activated cell sorting (FACS) analysis. Cells transfected with the plus sense gene bound greater quantities of this mAb than did the parental BHK cells or BHK cells transfected by the vector only (Fig. 7). Conversely, cells transfected with the gene in the antisense orientation exhibited a detectable decrease in the binding of mAb 1C3. Thus the concentration of laminin receptor expressed on the cell surface is correlated with the efficiency with which the cells can be infected by Sindbis virus, and we conclude that this receptor functions as a major receptor for Sindbis virus, at least in BHK cells.

The Laminin Receptor Serves as a Sindbis Virus Receptor in Other Mammalian Cells

We wished to determine if the laminin receptor also serves as a receptor for Sindbis virus in cells of different origin. For this purpose we compared the ability of mAb 1C3 to inhibit plaque formation by Sindbis virus in BHK (hamster), Vero (monkey), SW13 (human), and CEF (chicken). In preliminary experiments, the relative susceptibility of these cells to Sindbis virus was ascertained (Table 4). CEF were the most susceptible followed by BHK, Vero, and SW13. Note that SW13 cells were less than 1% as susceptible to the virus as were BHK cells.

Inhibition of plaque formation by 1C3 in these various cells is shown in Fig. 8 Plaque formation in all 3 mammalian cell lines was inhibited by this antibody, indicating that the laminin receptor is a major Sindbis receptor in all 3 lines and, presumably, in all mammalian cells. The antibody has no effect on CEF, however. The virus either uses a different receptor in chicken cells or attachment of the virus to the chicken laminin receptor is not blocked by mAb 1C3.

Immunoprecipitation of the Laminin Receptor from Other Cells

We wished to determine whether 1C3 would immunoprecipitate the laminin receptor from other cell lines. As shown in Fig. 9, a 68 kDa protein was precipitable from mouse (N18) cells, hamster (BHK) cells, and monkey (Vero) cells. A 71 kDa protein was immunoprecipitated by mAb 1C3 from chicken cells, which is of interest in light of the observation that this monoclonal antibody does not block virus binding to chicken cells. Either the 71kDa protein, which is presumably the laminin receptor, does not serve as a Sindbis receptor in chicken cells, or binding of mAb 1C3 to it does not block virus binding.

Discussion

We have demonstrated that a cell adhesion molecule, the high affinity laminin receptor, serves as a major receptor for Sindbis virus in mammalian cells. The high affinity laminin receptor has a polypeptide backbone of 295 amino acids but is modified in an unknown fashion so that its apparent molecular weight estimated from acrylamide gel electrophoresis is 68 kDa. There are no potential sites for aspargine-linked carbohydrates in the amino acid sequence and the nature of the posttranslational modifications remains obscure. Based on its amino acid sequence the laminin receptor has a short N-terminal cytoplasmic domain, a putative transmembrane domain in the middle of the molecule, and a C-terminal extracellular domain which has abundant glutamic acid and aspartic acid residues and several repeated sequences. The high affinity laminin receptor mediates cell-extracellular matrix interactions as well as cell-cell adhesion, and is important in tumor cell invasion and metastasis (Liotta et al., 1986). The laminin receptor also plays a significant role in many important cellular events such as adhesion, morphology, spreading, migration

and differentiation. It is also of interest that a high affinity laminin receptor encoding a 50kDa protein has been identified in *Staphylococcus aureus* (Lopes et al., 1985). These laminin receptors or proteins related to laminin receptors are widely distributed phylogenically.

Sindbis virus infects cultured cells from very widespread organisms, including mammals, birds, and mosquitoes (Chamberlain, 1980; Niklasson, 1989). We have tested a number of cell lines from lower vertebrates, bluegill fish trunk BF-2 cells, brown bullhead fish trunk BB cells, viper spleen VSW cells, and *Xenopus* kidney A6 cells, and found that the virus would infect all of these cells (KSW, unpublished data). It remains to be determined if the high affinity laminin receptor functions as a Sindbis virus receptor in all these cells, or whether it serves primarily or exclusively in mammalian cells and other molecules fulfill this function in other hosts.

The high affinity laminin receptor binds to a YIGSR sequence in the central region of laminin. This sequence is not found in the Sindbis glycoproteins and, as noted above, neither laminin nor the synthetic peptide YIGSR blocks virus binding to BHK cells. This suggests that the sites in the laminin receptor to which laminin binds and that to which Sindbis virus binds are distinct and spatially separated. From the overlap of the λgt11 clones to which mAb 1C3 binds, this mAb must bind somewhere in the C-terminal 48 amino acids of the laminin receptor. It seems likely that Sindbis virus also binds within or close to this same domain, since mAb 1C3 blocks binding of Sindbis virus, although steric hindrance of virus binding to other regions of the molecule or conformational rearrangements caused by antibody binding that interfere with virus attachment cannot be ruled out.

There are indications that a domain in glycoprotein E2 of Sindbis virus from residues 172 to 222 might form the virus antireceptor that binds to the cellular receptor (in this case the laminin receptor in mammals). This domain has been shown to be immunologically important, and many neutralization escape variants map to this region (Strauss et al., 1991). We have found that one neutralizing mAb will react with a linear determinant in this domain (Chapter 4), and that antiidiotypic antibodies to several

neutralizing mAbs that are reactive with this domain function as antireceptor antibodies (Chapter 3). Finally, it has been found that single amino acid changes in this region can result in altered affinities of the virus for different cells (Tucker and Griffin, 1991).

The relationship between the protein immunoprecipitated from chicken cells by antiidiotypic antibody 49 (Chapter 3) and the 71 Kd protein immunoprecipitated by mAb 1C3 remains to be determined. It is also uncertain as to whether the laminin receptor functions as a receptor for Sindbis virus in chicken cells. It is possible that the binding sites for 1C3 and for Sindbis virus differ, and that while binding of 1C3 to the mammalian receptor blocks Sindbis binding, binding of 1C3 to the chicken receptor does not because of differences in the primary amino acid sequences or on the posttranslational modifications effected in the two systems.

We have clearly shown that BHK cells that express different numbers of laminin receptors at their surface differ in the efficiency with which they are infected by Sindbis virus. It will be of interest to determine if the differential efficiencies with which different cells are infected by the virus, e.g., SW13 cells versus BHK cells, or cells of neuronal origin that are differentially susceptible to neurotropic strains of the virus, arise because these cells produce different concentrations of laminin receptor. It is also possible that the differential susceptibility might arise from differential posttranslational modifications of the laminin receptor in different cells, or from the availability of other (as yet unidentified) Sindbis receptors on these cells.

Although the data strongly support the hypothesis that the laminin receptor itself function directly as a Sindbis virus receptor, can not rule out the possibility that laminin receptor is only an accessory protein closed associated with actual Sindbis virus receptor. In this model, mAb 1C3 bound to laminin receptor would hinder virus attachment to an associated Sindbis virus receptor, and overexpressed laminin receptors or underexpressed laminin receptors from the transfectants is correlated with the actual virus receptor expression level. We consider this model unlikely.

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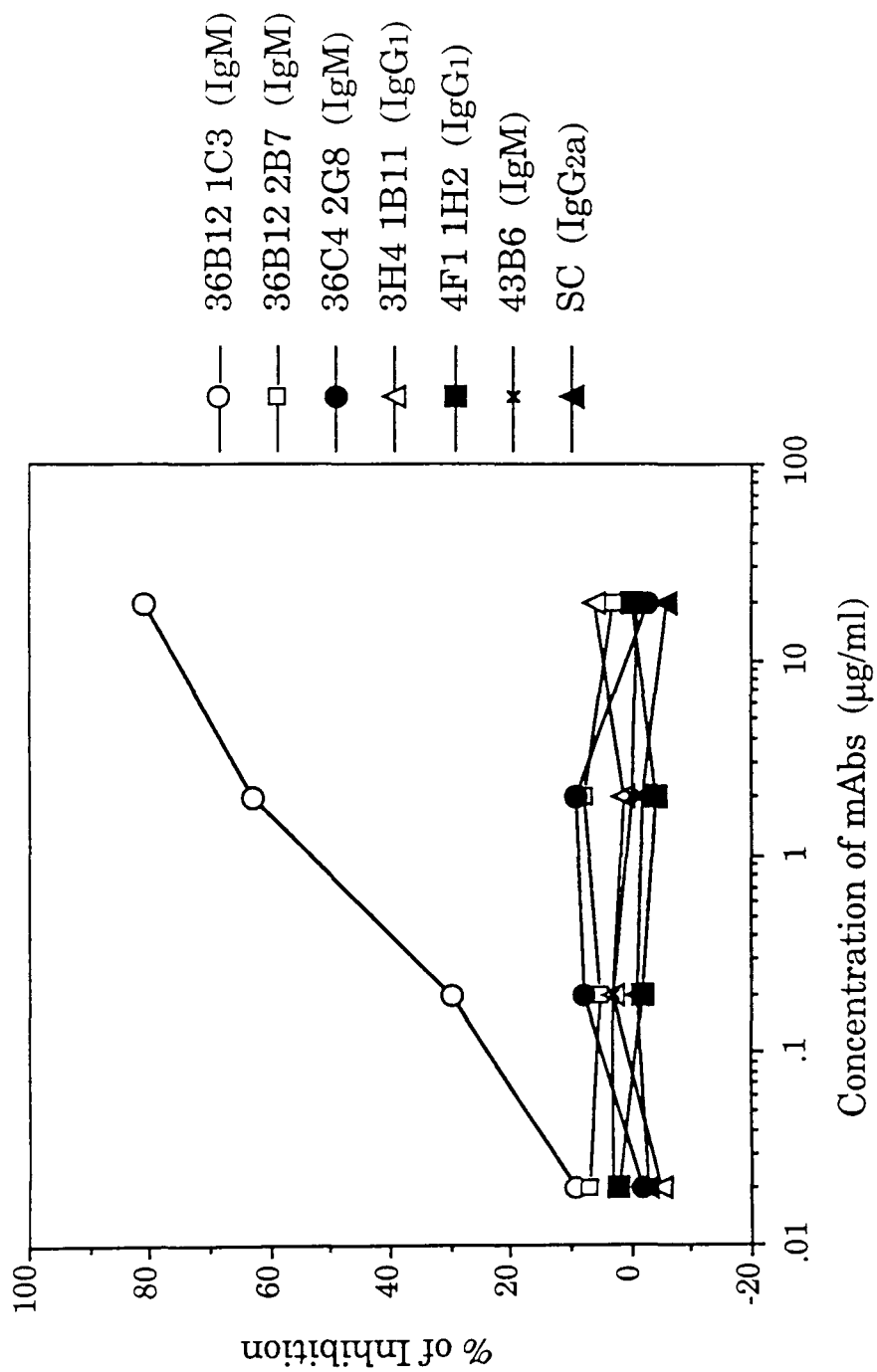
36B12 1C3 (IgM)
36B12 2B7 (IgM)
36C4 2G8 (IgM)
3H4 1B11 (IgG1)
4F1 1H2 (IgG1)
43B6 (IgM)
SC (IgG2a)
% of Inhibition
100
80
60
40
20
0
-20
.01
.1
1
10
100
Concentration of mAbs (µg/ml)

Fig. 1. Inhibition of Sindbis virus infection by anti-BHK monoclonal antibodies.

36B12 1C3 (IgM), 36B12 2B7 (IgM), 36C4 2G8 (IgM), 3H4 1B11 (IgG_1), and 4F1 1H2 (IgG_1) are various mAbs derived from mice immunized with whole BHK cells. 43B6 (IgM) is a monoclonal α–Id to anti-Sindbis glycoprotein E2 mAb 49, and SC (IgG_1) is an anti-rat membrane protein mAb. These mAbs were affinity purified and their ability to block Sindbis virus infection as assayed by plaque assay was compared at 0.02 μg/ml, 0.2 μg/ml, 2 μg/ml, and 20 μg/ml.

METHOD: Six-week old Balb/c mice were immunized i.p. with 10^7 whole BHK cells harvested from monolayer culture with 4 mM EDTA and mixed with adjuvant. Spleens were removed and lymphocytes fused with the myeloma cell line NS-1 using standard methods (Harlow and Lane, 1988). Two to three weeks later the supernatant fluids from wells with vigorously growing hybridoma colonies were assayed for their ability to block the infection of Sindbis virus strain AR339 in a plaque assay in monolayers of BHK cells in 12-well cluster plates as previously described (Wang et al., 1991).

To prepare purified antibody, the hybridoma producing mAb 1C3, and several control hybridomas were injected i.p. into athymic NCR six-week-old female nude mice (Simonsen Laboratory). Mice were first primed for one week by injection with pristane (2,6,10,14-tetramethyldecanoic acid), followed by injection with the mAb. Ascites fluids were collected after 7-10 days, and the mAbs purified from the ascites fluids by affinity chromatography. mAbs known to be IgM from Ouchterlony immunodiffusion assays were purified by affinity chromatography on rat anti-mouse IgM coupled to Sepahrose 4B (Zymed), and those known to be IgG were purifed on protein G sepharose columns (Pharmacia). The concentrations of the mAbs were determined by Lowry assay (Biorad) and by optical density at 280 nm.

Monolayers were incubated with the indicated amounts of purified mAbs in 180 µl of buffer for 1 hr at 22°C followed by addition of 10-150 PFU of Sindbis virus AR339 purified from sucrose gradients. After a further 1 hr incubation at 22°C, the incubation buffer was removed and replaced with Eagle's medium containing 1% agar. Plaques were visualized by staining with 1% neutral red after 18 hr at 37°C.

Table 1. Competition between ^{35}S-labeled Sindbis virus and mAbs for binding to BHK cells

mAb(isotype)[a]	Virus Bound (% of Control)[b]		
	0.2μg/ml[c]	2μg/ml	20μg/ml
36B12 1C3 (IgM)	79	53	28
36B12 2B7 (IgM)	91	95	94
36C4 2G8 (IgM)	102	109	96
3H4 1B11 (IgG1)	105	93	99
43B6 (IgM)	98	88	94
SC (IgG2a)	91	95	104

[a] Antibodies used have been described in the legend to Fig. 1.

[b] The amount of ^{35}S-labeled Sindbis virus bound to BHK cells expressed as a percent of control. BHK cells were seeded at $2x10^4$ per well in 96-well cluster plates, and grown at 37°C for 15 hr until confluent. The monolayers were then treated with different concentrations of the indicated mAb in a total volume of 25 μl PBS containing 1% fetal calf serum and 1 mM $MgCl_2$, for 1 hr at 10°C. Twenty-five μl of a solution of ^{35}S-labeled Sindbis virus (at 5649 cpm/μl) purified by sedimentation in a sucrose gradient was then added to each well, and incubation continued for 1.5 hr. The monolayers were washed, dissolved in 0.5% SDS, and bound radioactivity determined by liquid scintillation counting. The amount of labeled virus applied was sufficient to saturate the cell monolayers, and the amount of virus bound in the absence of mAb was taken as 100%.

[c] Concentration of mAb.

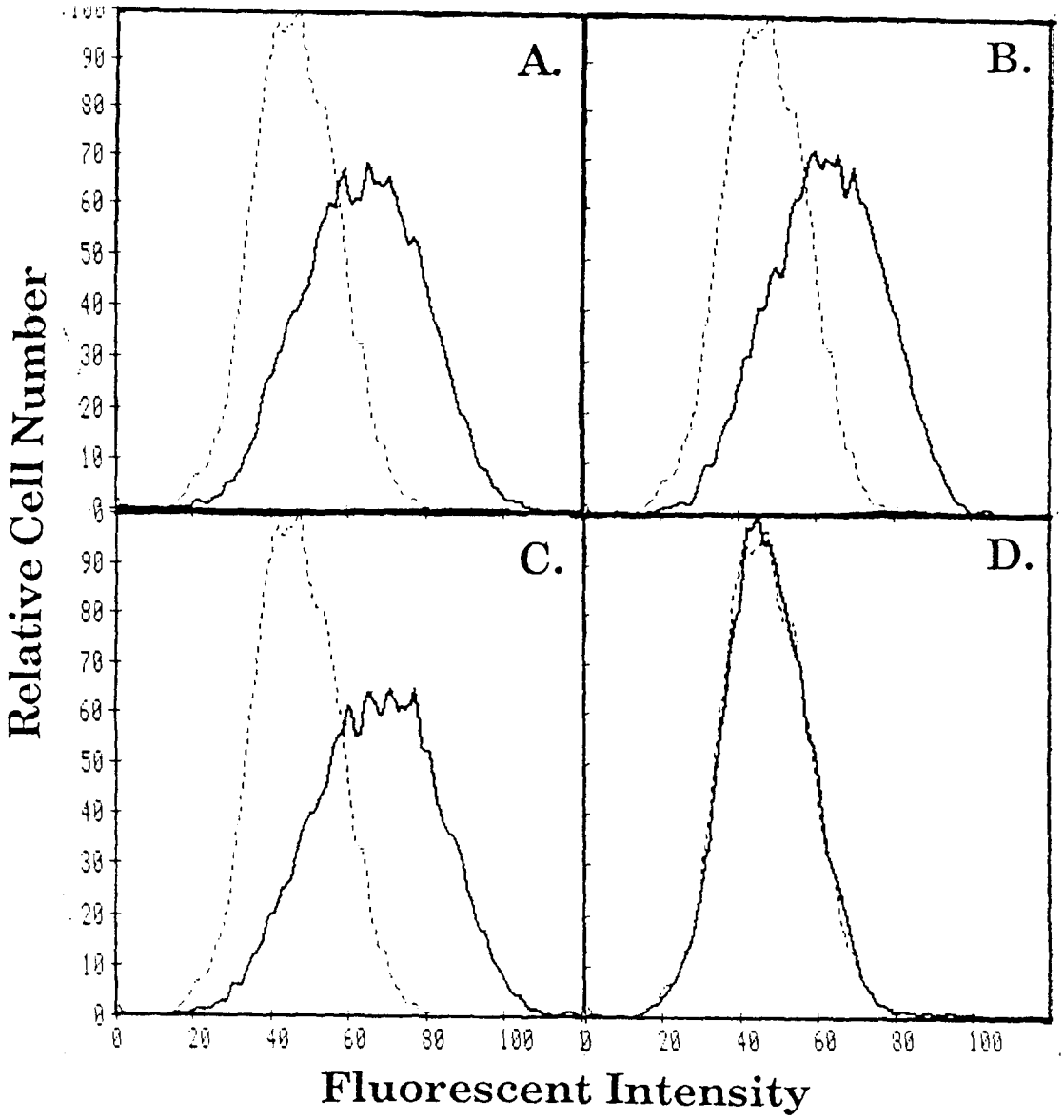
A.
B.
C.
D.
Relative Cell Number
Fluorescent Intensity
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100

Fig. 2 Binding of mAbs to the BHK cell surface

In each panel, the dashed line is the profile of BHK cells stained with secondary antibody FITC-conjugated goat anti-mouse IgM (μ chain) as a control. In panel A, the solid line is BHK cells stained with mAb 36B12 1C3, in panel B, anti-BHK mAb 36B12 2B7, in panel C, anti-BHK mAb 36C4 2G8, panel D, anti-mouse IgG mAb 43B6.

METHOD:

Monolayers of BHK cells were washed with PBS lacking divalent cations and removed from the culture dish by treatment with 4mM EDTA in PBS for 15 min at 37°C. 10^6 cells were incubated in 400μl PBS containing 1% FCS, 100μg/ml DNase I and 10μg of each mAb for 40 min at 4°C. The cells were washed, 400μl of a 1:100 dilution of FITC-conjugated goat anti-mouse IgM (μ chain) (Cappel) was added as the secondary antibody, and the mixture incubated for an additional 40 min at 4°C. The washed cells were resuspended in 1 ml PBS containing 1% FCS, and filtered through a nylon screen (Tetko Instruments). 5μl of 10μg/ml propidium iodide (Sigma) was added to enable of nonviable cells to be electronically gated out (Boyer et al. 1988) and flow cytometric analysis was performed (Ormerod, 1990).

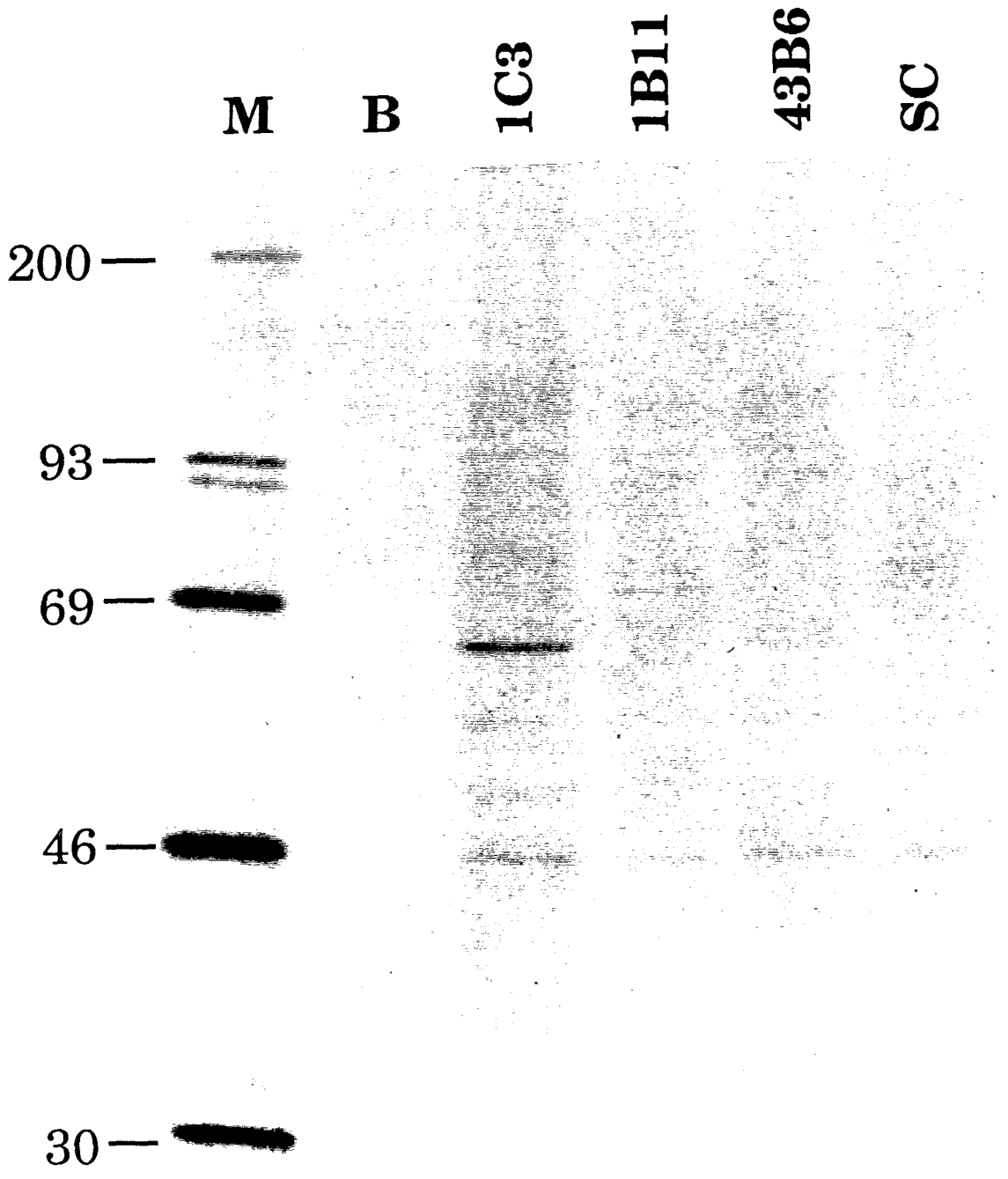
M
B
1C3
1B11
43B6
SC
200
93
69
46
30

Fig. 3 Immunoprecipitation by mAb 1C3 of a 68 Kd protein from ^{35}S-labeled membrane preparations from BHK cells.

Membrane preparations from ^{35}S-labeled BHK cells were immunoprecipitated as previously described (Hardy and Strauss, 1988), with 5 μg of the mAb indicated; the mAbs are the same as in Table 1. Immunoprecipitates in lanes B (control with no mAb added), 1C3, and 43B6 were collected by treatment with rat anti-mouse IgM coupled to sepharose 4B (Zymed). Immunoprecipitates with 1B11 and SC were collected by treatment with protein G sepharose (Pharmacia). Labeled molecular weight standards are shown in lane M.

METHOD:

BHK cells were grown in 800 cm^2 roller bottles and labelled with 25 μC/ml [^{35}S] Met (Amersham) for 12 hr. Cell monolayers were washed with PBS, removed from the glass with 4mM EDTA, collected by centrifugation and lysed by Dounce homogenization in 1 mM $NaHCO_3$, 10 mM EDTA and protease inhibitors (1 mM PMSF, 0.7 μg/ml pepstatin A, and 0.5 μg/ml leupeptin). The lysate was centrifuged at 120,000 g for 1 hr in the SW55Ti rotor, and the pellet resuspended by Dounce homogenization in 1 ml of 53% (w/w) sucrose in 10 mM $NaPO_4$ (pH 7.0). The resuspended pellet was placed in a 5 ml nictrocellulose centrifugation tube and overlaid with 1 ml aliquots of 41%, 37%, 33%, and 8.5% (w/w) sucrose solutions in the same buffer. This stepwise gradient was centrifuged in the SW55Ti rotor for 20 hr at 120,000g. One ml fractions were collected from the top of the gradient, mixed with 1 ml of binding buffer (50 mM Tris-Cl, pH 7.5, 0.3 M NaCl, 4 mM EDTA, containing 0.5% triton X-100, 200μg/ml bovine serum albumin and the protease inhibitors described above).

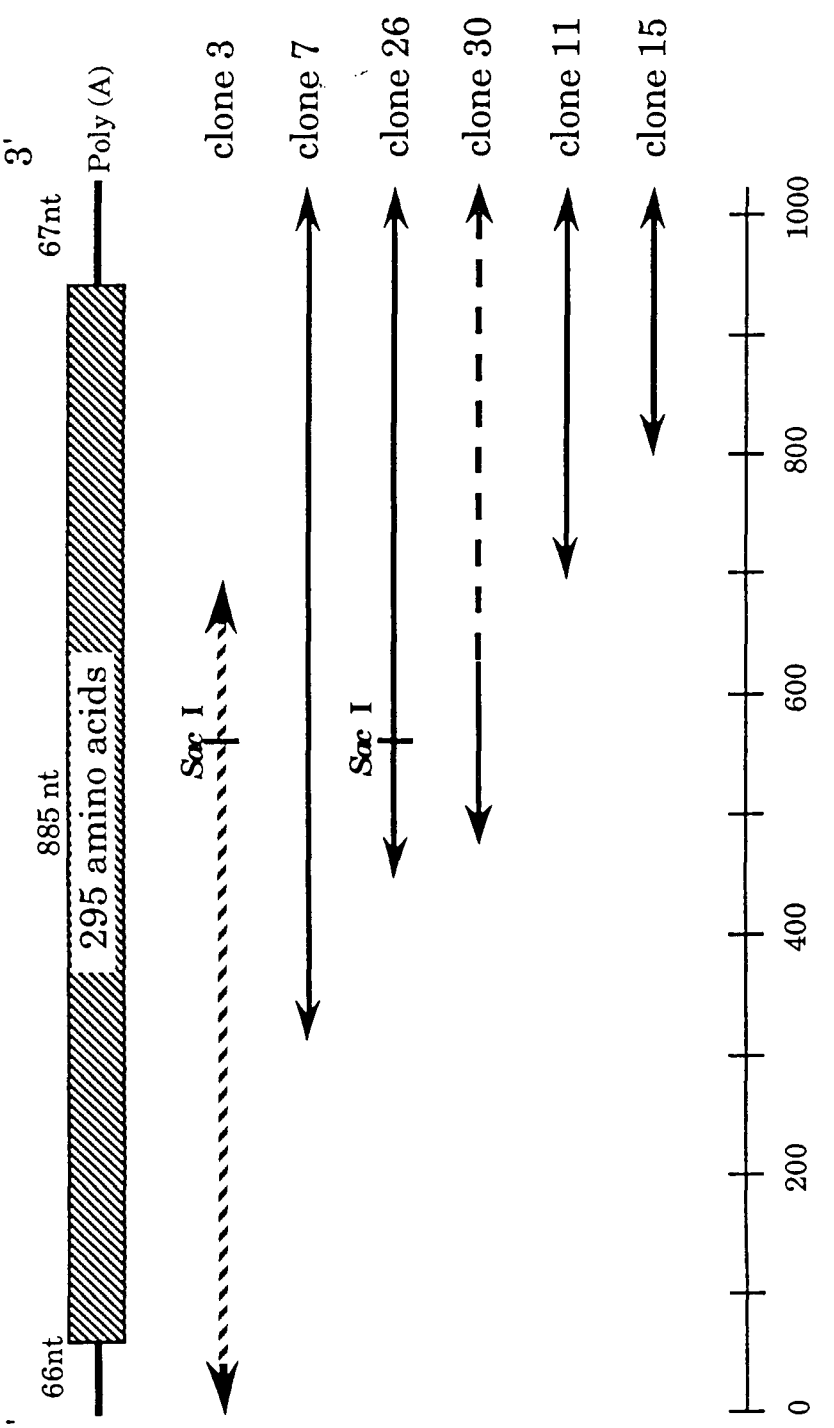
5'
66nt
885 nt
295 amino acids
67nt
Poly (A)
3'
clone 3
clone 7
clone 26
clone 30
clone 11
clone 15
Sac I
Sac I
0
200
400
600
800
1000

Fig. 4. Map of λgt11 clones containing BHK laminin receptor cDNA inserts.

The open box depicts the coding region of the BHK laminin receptor. Five clones selected by their immunoreactivity with mAb 1C3 are illustrated to scale. The λgt11 clone containing the 5' end of the gene (clone 3, dashed line) was selected by screening with a probe from clone 26.

METHOD: A λgt11 cDNA expression library was constructed from oligo(dT) primed cDNA made to poly(A)-selected RNA from BHK cells. Lambda plaques were screened with mAb 1C3 as the primary antibody and ^{125}I-conjugated goat anti-mouse IgM as secondary antibody. Fifteen reactive clones were isolated from 10^6 λgt11 plaques. Six of the 15 clones were found to cross hybridize, and the sequences of five of these were determined; they were found to start at various points in the laminin receptor sequence and terminate with the 3' poly(A).

To isolate clones containing the 5' end of the sequence, the insert from clone 26 was excised with *Eco*RI, labelled by nick translation with [^{32}P]dCTP, and used to screen the λgt11 library for clones which overlapped the clone 26 sequence. Out of 5 x 10^4 λgt11 plaques, 124 reactive clones were found. Of these 52 were analyzed by PCR for the 5' end of the laminin receptor coding region, using the 17-mer oligonucleotide primer 5'-GCAAACTTCAGCACAGC-3' complementary to the 5' end of clone 7 and either the 24-mer λgt11 forward primer or the 24-mer λgt11 reverse primer. Five μl of lambda phage solution from a 200 μl suspension of a single plaque in 2.5 mM $MgCl_2$, 2.5 mM TrisCl pH 8.0, and 0.01% gelatin were amplified with 10 pmol of the primers in a volume of 50 μl of PCR buffer and 1.5 units Taq polymerase (Promega) (94°C for 3 min, 35 cycles of 1 min at 94°C, 1.5 min at 55°, and 2 min at 72°C, then 72° for 7 min). Amplified inserts were characterized by eletrophoresis and DNA sequencing; clone 3 was identified as encoding the 5' end of the laminin receptor.

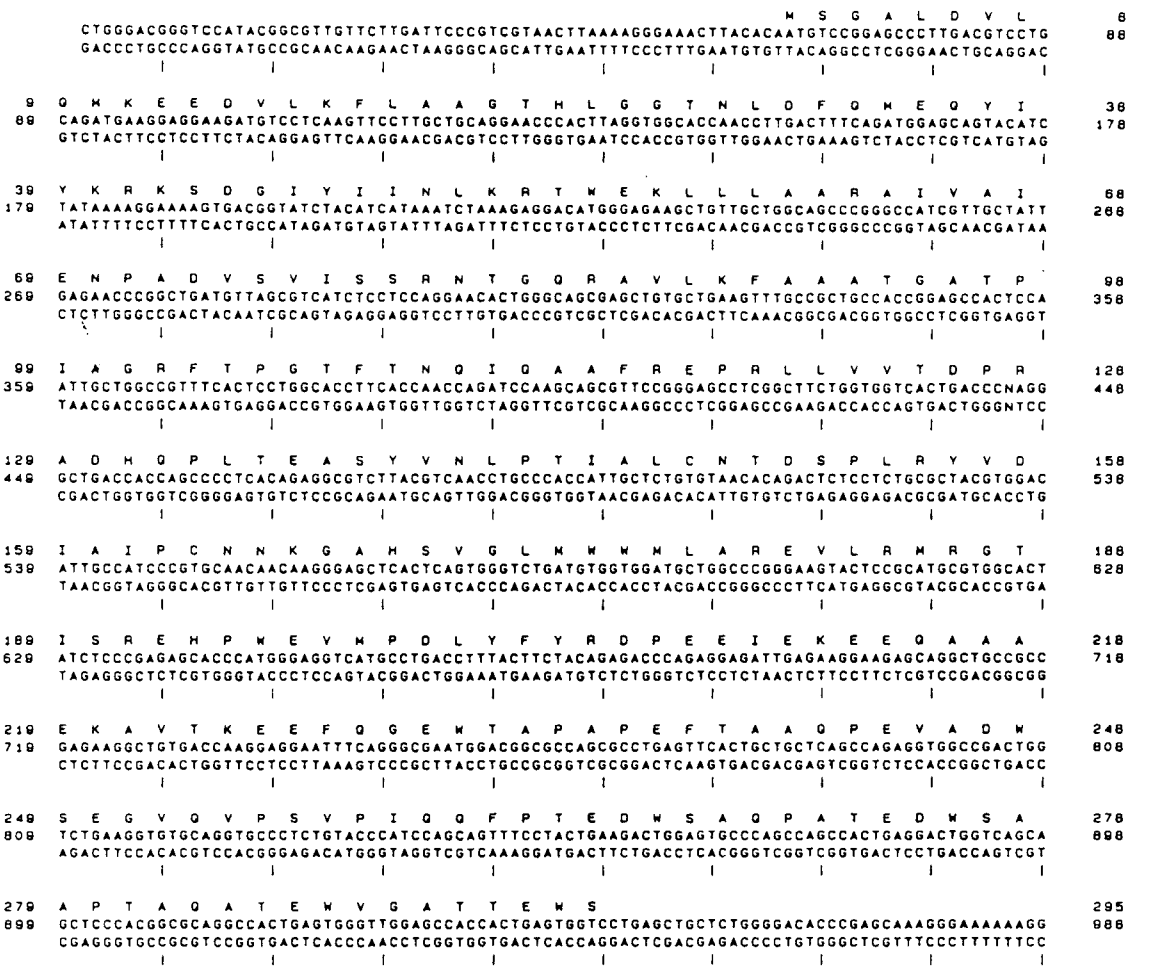
M S G A L D V L 8
CTGGGACGGGTCCATACGGCGTTGTTCTTGATTCCCGTCGTAACTTAAAAGGGAAACTTACACAATGTCCGGAGCCCTTGACGTCCTG 88
GACCCTGCCCAGGTATGCCGCAACAAGAACTAAGGGCAGCATTGAATTTTCCCTTTGAATGTGTTACAGGCCTCGGGAACTGCAGGAC
9 Q M K E E D V L K F L A A G T H L G G T N L D F Q M E Q Y I 38
88 CAGATGAAGGAGGAAGATGTCCTCAAGTTCCTTGCTGCAGGAACCCACTTAGGTGGCACCAACCTTGACTTTCAGATGGAGCAGTACATC 178
GTCTACTTCCTCCTTCTACAGGAGTTCAAGGAACGACGTCCTTGGGTGAATCCACCGTGGTTGGAACTGAAAGTCTACCTCGTCATGTAG
39 Y K R K S D G I Y I I N L K R T M E K L L L A A R A I V A I 68
178 TATAAAAGGAAAAGTGACGGTATCTACATCATAAATCTAAAGAGGACATGGGAGAAGCTGTTGCTGGCAGCCCGGGCCATCGTTGCTATT 268
ATATTTTCCTTTTCACTGCCATAGATGTAGTATTTAGATTTCTCCTGTACCCTCTTCGACAACGACCGTCGGGCCCGGTAGCAACGATAA
69 E N P A D V S V I S S R N T G Q R A V L K F A A A T G A T P 98
268 GAGAACCCGGCTGATGTTAGCGTCATCTCCTCCAGGAACACTGGGCAGCGAGCTGTGCTGAAGTTTGCCGCTGCCACCGGAGCCACTCCA 358
CTCTTGGGCCGACTACAATCGCAGTAGAGGAGGTCCTTGTGACCCGTCGCTCGACACGACTTCAAACGGCGACGGTGGCCTCGGTGAGGT
99 I A G R F T P G T F T N Q I Q A A F R E P R L L V V T D P R 128
359 ATTGCTGGCCGTTTCACTCCTGGCACCTTCACCAACCAGATCCAAGCAGCGTTCCGGGAGCCTCGGCTTCTGGTGGTCACTGACCCNAGG 448
TAACGACCGGCAAAGTGAGGACCGTGGAAGTGGTTGGTCTAGGTTCGTCGCAAGGCCCTCGGAGCCGAAGACCACCAGTGACTGGGNTCC
129 A D H Q P L T E A S Y V N L P T I A L C N T D S P L R Y V D 158
449 GCTGACCACCAGCCCCTCACAGAGGCGTCTTACGTCAACCTGCCCACCATTGCTCTGTGTAACACAGACTCTCCTCTGCGCTACGTGGAC 538
CGACTGGTGGTCGGGGAGTGTCTCCGCAGAATGCAGTTGGACGGGTGGTAACGAGACACATTGTGTCTGAGAGGAGACGCGATGCACCTG
159 I A I P C N N K G A H S V G L M W W M L A R E V L R M R G T 188
539 ATTGCCATCCCGTGCAACAACAAGGGAGCTCACTCAGTGGGTCTGATGTGGTGGATGCTGGCCCGGGAAGTACTCCGCATGCGTGGCACT 628
TAACGGTAGGGCACGTTGTTGTTCCCTCGAGTGAGTCACCCAGACTACACCACCTACGACCGGGCCCTTCATGAGGCGTACGCACCGTGA
189 I S R E H P W E V M P D L Y F Y R D P E E I E K E E Q A A A 218
629 ATCTCCCGAGAGCACCCATGGGAGGTCATGCCTGACCTTTACTTCTACAGAGACCCAGAGGAGATTGAGAAGGAAGAGCAGGCTGCCGCC 718
TAGAGGGCTCTCGTGGGTACCCTCCAGTACGGACTGGAAATGAAGATGTCTCTGGGTCTCCTCTAACTCTTCCTTCTCGTCCGACGGCGG
219 E K A V T K E E F Q G E W T A P A P E F T A A Q P E V A D W 248
719 GAGAAGGCTGTGACCAAGGAGGAATTTCAGGGCGAATGGACGGCGCCAGCGCCTGAGTTCACTGCTGCTCAGCCAGAGGTGGCCGACTGG 808
CTCTTCCGACACTGGTTCCTCCTTAAAGTCCCGCTTACCTGCCGCGGTCGCGGACTCAAGTGACGACGAGTCGGTCTCCACCGGCTGACC
249 S E G V Q V P S V P I Q Q F P T E D W S A Q P A T E D W S A 278
809 TCTGAAGGTGTGCAGGTGCCCTCTGTACCCATCCAGCAGTTTCCTACTGAAGACTGGAGTGCCCAGCCAGCCACTGAGGACTGGTCAGCA 898
AGACTTCCACACGTCCACGGGAGACATGGGTAGGTCGTCAAAGGATGACTTCTGACCTCACGGGTCGGTCGGTGACTCCTGACCAGTCGT
279 A P T A Q A T E W V G A T T E W S 295
899 GCTCCCACGGCGCAGGCCACTGAGTGGGTTGGAGCCACCACTGAGTGGTCCTGAGCTGCTCTGGGGACACCCGAGCAAAGGGAAAAAAGG 988
CGAGGGTGCCGCGTCCGGTGACTCACCCAACCTCGGTGGTGACTCACCAGGACTCGACGAGACCCCTGTGGGCTCGTTTCCCTTTTTTCC
989 TGGAAGGAAAATAAAGTTTCTACAAGCTG 1017
ACCTTCCTTTTATTTCAAAGATGTTCGAC

Fig. 5 Nucleotide sequence and deduced amino-acid sequence of the BHK laminin receptor. The nucleotide sequence determined from the clones shown in Figure 3 is shown. The sequence contains a single open reading frame which begins at the methionine codon at nucleotides 65-67, and terminates at the stop codon at nucleotides 950-952. A published amino acid sequence (Makrides et al., 1988) for the mouse high affinity laminin receptor is identical to that for BHK shown here. There are two amino acid differences between the BHK sequence and the sequence of the human laminin receptor (Wewer et al., 1986; Yow et al., 1988): Ala-241$_{BHK,mouse}$ --> Thr-241$_{human}$ and Glu-293 $_{BHK,mouse}$ --> Asp-293 $_{human}$. A second published sequence for the mouse laminin receptor (Rao et al., 1989) differs in two amino acids from the sequence published by Makrides et al. (1988).

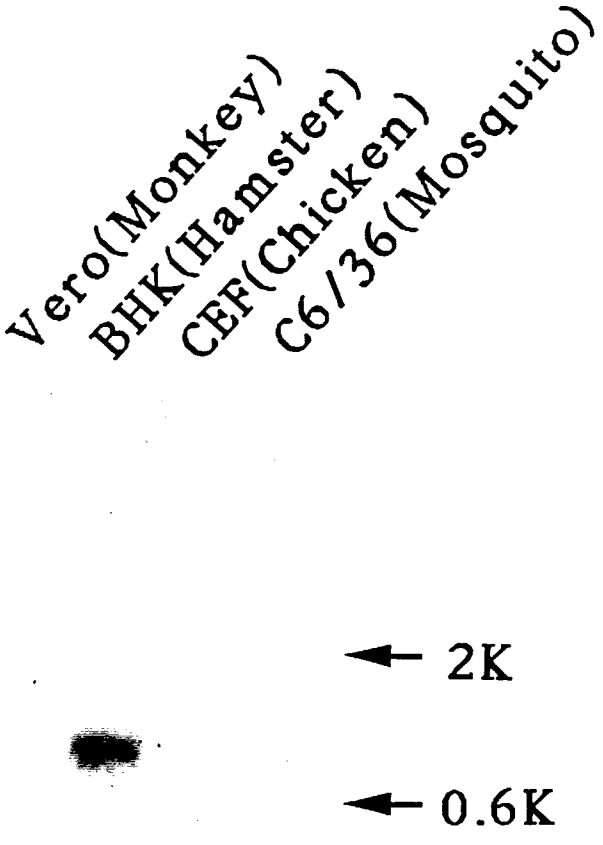
Vero(Monkey)
BHK(Hamster)
CEF(Chicken)
C6/36(Mosquito)
2K
0.6K

Fig. 6 Northern blot analysis of mRNA extract from distinct several cell lines.

Expression of laminin receptor mRNA was analyzed by using the 3' end of BHK laminin receptor cDNA from λ clone 26 to make a ^{32}P- labeled probe. Cell lines examined were mosquito C6/36 cells, chicken embryo fibroblast cells, hamster BHK cells and monkey Vero cells.

Method:

1. The EcoRI fragment encoding 3' end of BHK laminin receptor of λ clone 26 was used to make 32P-dCTP labeled probes by nick translation (Sambrook et al. 1989).

2. mRNA was prepared from culture cells using the guanidinium thiocyanate-LiCl method. 5μg of each mRNA was subjected to formaldehyde agarose gel electrophoresis and transfered to nitrocellulose membrane (Satorius). The hybridization procedure is from Sambrook et al. (1989). The filters were washed at low stringency in 2xSSC, 50°C, and exposed to X-ray film for 2 days in the presence of an intensifying screen at -80°C.

Table 2. Efficiency of plaque formation by Sindbis virus in BHK cells transfected with the laminin receptor gene or with the anti-sense gene

Clones transfected with the laminin receptor gene[a]	
Number of clones	Relative efficiency of plaque formation[b]
14	4 - 5 fold
39	3 - 4 fold
14	2 - 3 fold
6	1 - 2 fold

Clones transfected with antisense receptor [c]	
Number of clones	Relative efficiency of plaque formation
31	0.35 - 0.5 fold
12	0.5 - 0.7 fold
14	0.7 - 0.9 fold
8	0.9 - 1.0 fold

[a] Cells were transfected with the construct LR cDNA-1 in which a full-length BHK laminin receptor cDNA (sequence shown in Fig. 4) flanked by *Eco*RI linkers was inserted into the *Eco*RI site in the polylinker of the pcDNA1/neo expression vector (Introgene).

[b] BHK cells were transfected with 5 μg of either the plus sense or anti-sense construct in the pcDNA1/neo vector or with the vector alone using the lipofectin (BRL) transfection method (Felgner et al., 1987). After 24 hr the cells were placed in medium containing 400 μg/ml Geneticin (Gibco), and the Geneticin-resistant colonies were selected 2 weeks after transfection. Transfectants were screened for plaquing efficiency of Sindbis virus AR339.

c The anti-sense LR cDNA-7 construct contains the same insert as above in the opposite orientation in the pcDNA-1/neo vector.

Table 3. Sindbis Virus Plaque Formation on pcDNA1/neo Transfected BHK

Cell Lines[a]	Plaques % of Control
BHK (nontransfected)	**100**
BHK transfected with pcDNA1/neo	
Vector only	**105**
+ sense laminin receptor	
clone 33	**412**
clone 40	**385**
clone 41	**440**
clone 52	**407**
antisense laminin receptor[b]	
clone 4	**40**
clone 6	**45**
clone 38	**42**

[a] Four independent clones (#33, #40, #41, and#52) were chosen from 73 clones of BHK cells transfected with the sense construct and three independent transfectants (#4, #6, and #38) were selected from 65 clones transfected with the anti-sense laminin receptor (see Table 2). Nontransfected BHK cells and cells transfected with the pcDNA-1/neo vector only were used as controls.

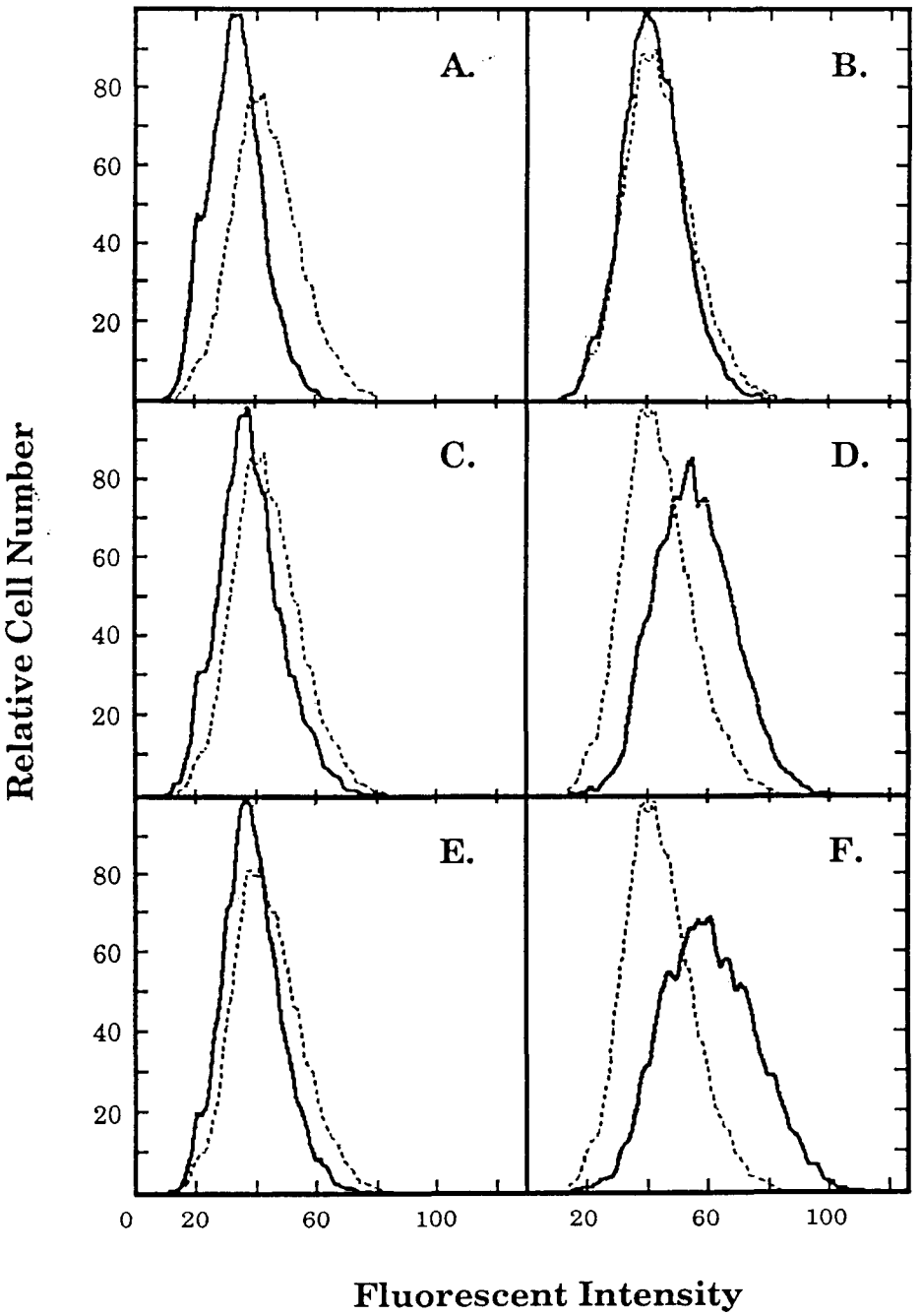
A.
B.
C.
D.
E.
F.
80
60
40
20
0
20
60
100
Relative Cell Number
Fluorescent Intensity

Fig. 7 Binding of mAb 1C3 to the laminin receptor transfectant BHK cells as determined by FACS analysis.

In each panel, the dashed line is the profile of BHK cells transfected with the pcDNA/neo vector alone and stained with mAb 1C3. In Panel A, the solid line is nontransfected cells stained with the secondary antibody alone; in all other panels the cells have been stained with mAb1C3. Panel B, nontransfected BHK cells. Panel C, clone 4 LR antisense transfected BHK. Panel D, clone 41 LR sense transfected. Panel E, clone 38 LR antisense transfected. Panel F, clone 52 LR sense transfected.

METHOD: Monolayers of BHK cells were washed with PBS lacking divalent cations and removed from the culture dish by treatment with 4 mM EDTA in PBS for 15 min at 37°C. 10^6 cells were incubated in 400 µl PBS containing 1% FCS, 100 µg/ml DNase I and 10 µg mAb 1C3 for 40 min at 4 °C. The cells were washed, 400 µl of a 1:100 dilution of FITC-conjugated goat anti-mouse IgM (µ chain) (Cappel) was added as the secondary antibody, and the mixture incubated for an additional 40 min at 4°C. The washed cells were resuspended in 1 ml PBS containing 1% FCS, and filtered through a nylon screen (Tetko Instruments). 5 µl of 10 µg/ml propidium iodide (Sigma) was added to enable of nonviable cells to be electronically gated out (Boyer and Rothenberg, 1988) and flow cytometric analysis was performed (Ormerod, 1990).

Table. 4

Susceptibility of Infectible cells to Sindbis Virus

Cell lines	[a]susceptibility
CEF(Chicken)	~200
BHK(Hamster)	100
Vero(Monkey)	~50
SW13(Human)	~0.6

[a]We set plaque formation number appeared in BHK monolayers as 100% with same diluted virus

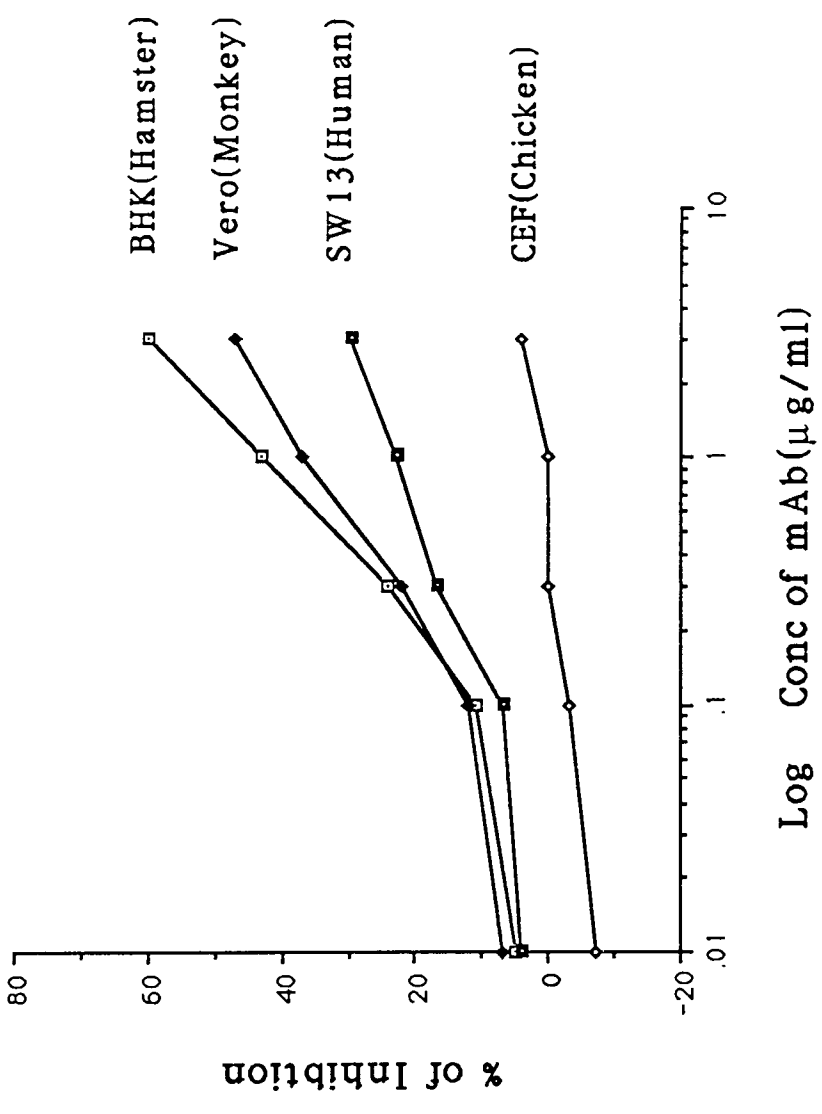
BHK(Hamster)
Vero(Monkey)
SW13(Human)
CEF(Chicken)
% of Inhibition
80
60
40
20
0
-20
.01
.1
1
10
Log Conc of mAb(µg/ml)

Fig.8 Inhibition of Sindbis virus infection by mAb 1C3 in different cells.

Five different concentrations of mAb 1C3 (0.01 μg/ml, 0.1 μg/ml, 0.3 μg/ml, 1 μg/ml and 3 μg/ml), were tested for inhibition of Sindbis virus infection in a plaque assay, as described in the legend to Fig. 1, on four different cell lines. Plaque counts have been normalized to the number of plaques on monolayers of the same cells without mAb treatment. Plaques were allowed to develop under 1% agarose in 12-well cluster plates for 18 hr in BHK cells, 24 hr in Vero cells, 32 hr in SW13 cells, and 13 hr in chicken cells before staining.

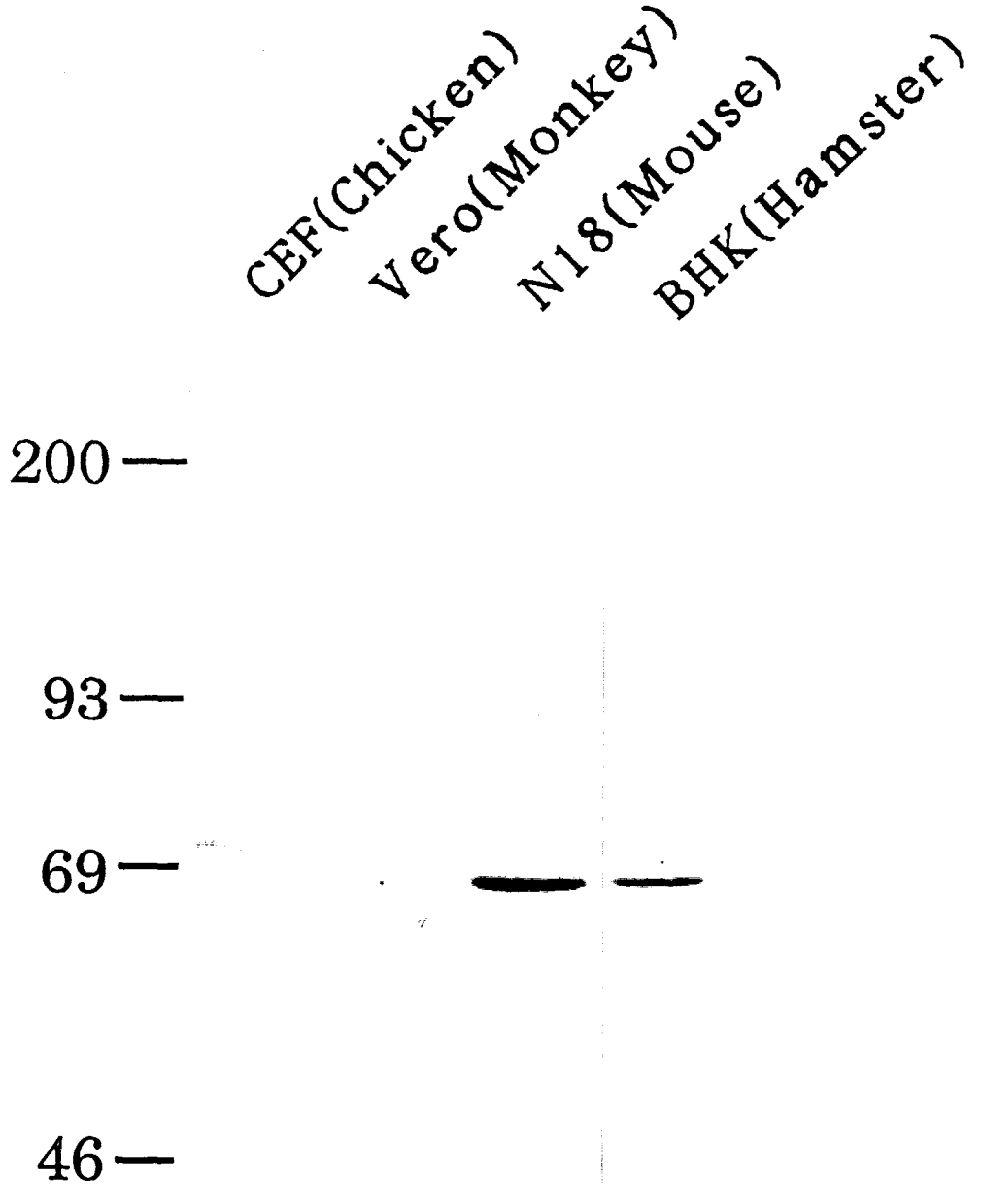
CEF(Chicken)
Vero(Monkey)
N18(Mouse)
BHK(Hamster)
200
93
69
46

Fig. 9 Immunoprecipitation of the laminin receptor from different cell lysates by mAb 1C3.

Cell lysates were prepared from ^{35}S-labeled mouse neuroblastoma cells, Vero cells, BHK cells, and chicken fibroblast cells, as indicated, and were immunoprecipitated with mAb 1C3. The immunoprecipitates were collected using goat anti-mouse IgM coupled to sepharose 4B.

METHOD: CEF, Vero, and BHK cells were grown in 800 cm^2 roller bottlesand labeled with 25 μCi/ml [^{35}S]Met (Amersham) for 12 hr. The cells were washed, removed from the bottle by treatment with 4 mM EDTA in PBS, and collected by centrifugation. Mouse N18 neuroblastoma cells were grown in T-150 flasks and labeled in the same way. Cell pellets were resuspended in 1 mM $NaHCO_3$ containing 10 mM EDTA and protease inhibitors (1 mM PMSF, 0.7 μg/ml pepstain A, 0.5 μg/ml leupeptin) and the cells broken by Dounce homogenization. A soluble membrane extract was prepared by centrifugation for 1 hr at 120,000 g and the pellet resuspended at 4°C in 50 mM Tris-Cl pH 7.5, 0.3 M NaCl, 200 μg/ml BSA, 4 mM EDTA, containing 3% Triton X-100 and protease inhibitors. Immunoprecipitations were performed as described (Hardy and Strauss, 1988), using goat anti-mouse IgM coupled to sepharose 4B to collect immunoprecipitates.

Chapter 3

Antiidiotypic Antibodies as Probes for the Sindbis Virus Receptor

This chapter was published in Virology

VIROLOGY 181, 694–702 (1991)

Antiidiotypic Antibodies as Probes for the Sindbis Virus Receptor

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Received November 16, 1990; accepted January 7, 1991

Rabbit polyclonal antiidiotypic antibodies were made to mouse monoclonal antibodies that neutralize the infectivity of Sindbis virus. One of the antiidiotypic antisera obtained has properties characteristic of an antireceptor antiserum. It binds to the surface of chicken cells as shown by immunofluorescence and partially blocks virus binding to these cells as determined by binding of radiolabeled virus or by a plaque reduction assay. It also immunoprecipitates a protein with a molecular weight of 63,000 from chicken cells. From the fact that the antiserum will only partially block virus uptake, and that it does not block uptake of a variant of Sindbis virus resistant to the monoclonal antibody used to produce the antiidiotypic antiserum, we propose that at least two distinguishable receptors can be used by Sindbis virus to enter chicken cells. Furthermore, the receptors used by Sindbis to enter BHK cells appear to be different from those on chicken cells, at least in part, in that the antiidiotypic antiserum does not recognize the BHK counterpart of the chicken cell receptor. We suggest that the alphaviruses use a number of distinguishable receptors which differ depending on the host and the tissue. In chicken cells the 63,000 molecular weight protein may be one of them. The diversity of such multiple receptors could account for the very wide host range of the alphaviruses, which infect mosquitoes, birds, and mammals.

INTRODUCTION

Animal viruses initiate infection of susceptible cells by binding to receptors expressed on the surfaces of cells, followed by entry into the cell. The distribution of these receptors determines, at least in part, the host range and tissue tropisms of viruses, since cells that do not express functional receptors are not susceptible to virus infection. The receptors are believed to influence the seriousness of the ensuing viral disease and at least in some cases it is thought that vaccine strains of viruses are altered in their receptor affinities and therefore infect a different subset of cells within the host. Protein receptors expressed on the surface of cells have been identified for HIV (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986), poliovirus (Mendelsohn *et al.*, 1989), the major human rhinovirus (Greve *et al.*, 1989; Staunton *et al.*, 1989), reovirus type 3 (Co *et al.*, 1985a,b), and Epstein–Barr virus (Fingeroth *et al.*, 1984; Frade *et al.*, 1985; Nemerow *et al.*, 1985). The first three receptors are all different members of the immunoglobulin superfamily. Although many viruses use protein receptors, at least some viruses use glycolipids, the best characterized being influenza (Weis *et al.*, 1988) and Sendai viruses (Markwell *et al.*, 1981).

The alphaviruses are a genus within the family Togaviridae that contains 26 members, many of which are important human pathogens. Many of the New World viruses such as Eastern, Western, and Venezuelan equine encephalitis viruses can cause encephalitis whereas the Old World viruses more typically lead to fever, rash and arthralgia (Griffin, 1986; Peters and Dalrymple, 1990). The alphaviruses are transmitted by insects and are therefore classified epidemiologically as arboviruses, being formerly called the Group A arboviruses. In almost all cases, the insect vector is a mosquito but in at least one case, Ft. Morgan virus, the vector is a swallow bug (Chamberlain, 1980). Birds and mammals form the primary vertebrate reservoirs of the viruses, but at least some members of the alphavirus family have been isolated from amphibians and reptiles as well (Chamberlain, 1980; Niklasson, 1989). Thus these viruses possess a very wide host range and it is an intriguing question as to whether they use the same receptor in all these divergent hosts, or whether the alphaviruses have evolved the ability to use different receptors in different organisms.

The alphaviruses are enveloped RNA-containing viruses in which an icosahedryl nucleocapsid is surrounded by a lipid-containing envelope containing two virus encoded glycoproteins, E2 and E1 (reviewed in Strauss and Strauss, 1986). The virus glycoproteins, which are present in the virus surface in hexameric spikes (Fuller, 1987), contain the virus attachment moiety as well as the neutralization epitopes of the

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0042-6822/91 $3.00

virus. Antiidiotypic antibodies (α-Ids) have been used in a number of systems as probes for receptors (reviewed in Gaulton and Greene, 1986). In an attempt to identify the receptors that function in Sindbis virus entry, we have isolated a series of α-Ids made to monoclonal antibodies (mAbs) that neutralize the infectivity of Sindbis virus, the prototype alphavirus, and screened these for their ability to block virus binding to susceptible cells. We have identified an α-Id that appears to be an antireceptor antibody.

MATERIALS AND METHODS

α-Id antibodies

Eight mAbs reactive with Sindbis virus were purified from mouse ascitic fluids by protein A chromatography, and were used to immunize New Zealand white female rabbits for the production of polyclonal α-Ids. Each rabbit was injected subcutaneously at multiple sites with 100 μg mAb in Freund's complete adjuvant, boosted at one month with 50 μg mAb in incomplete adjuvent, and bled 2 weeks after the second immunization. Unpurified sera had α-Id activities demonstrable by specific interactions with peroxidase-labeled mAbs, but also contained high levels of anti-immunoglobulin antibodies. Therefore, α-Ids were affinity purified, using conventional methods. In brief, sera were precipitated with ammonium sulfate, dialyzed against PBS (Dulbecco and Vogt, 1954), depleted of unwanted specificities by adsorption to isotype-matched "irrelevant" immunoglobulins (anti-dengue virus mAbs) covalently affixed to Sepharose 4B (Pharmacia), adsorbed to homologous (idiotypic) solid-phase mAb, eluted with 3.5 *M* NaSCN, dialyzed and sterilized by filtration.

Peroxidase-labeled (idiotypic) mAbs were used in the characterization of α-Ids, either by direct or competitive binding ELISAs similar to those described previously (Schmaljohn *et al.*, 1983). Typically 50 ng of α-Id in 50 μl PBS was added to wells of polystyrene EIA plates (Costar) and allowed to adsorb for 4 hr at room temperature. After removing the α-Id solution, the wells were blocked with 0.5% BSA containing 0.1% normal mouse serum. The adsorbed α-Id was then tested for its ability to bind peroxidase-labeled Ids. To evaluate possible shared idiotypy among mAbs, unlabeled mAbs were tested for their capacities to competitively inhibit interactions between α-Ids and their homologous peroxidase-labeled Ids.

RESULTS

Characteristics of α-Ids

A number of mAbs directed against the structural proteins of Sindbis virus have been previously characterized (Schmaljohn *et al.*, 1982, 1983; Stec *et al.*, 1986). Eight of these antibodies were inoculated into rabbits to elicit polyclonal α-Id sera to be used as probes for the study of the Sindbis virus receptor. Characteristics of the 8 mAbs used are shown in Table 1. Five were directed against glycoprotein E2 and neutralized virus infectivity. Two were reactive with glycoprotein E1, one of which neutralized virus infectivity whereas the other did not. Finally, a mAb reacting with the capsid protein was used in the study as an additional control. Each of the eight α-Ids reacted by ELISA with the mAb used for its induction. The interaction between each α-Id and the corresponding peroxidase-labeled mAb was competitively inhibited by homologous unlabeled mAb, and at 32 μg/ml inhibition was complete (Fig. 1 and data not shown). In contrast, for each α-Id, the seven heterologous unlabeled mAbs in Table 1 did not compete with homologous idiotypic mAb by more than 20% at 32 μg/ml (Table 2 and data not shown). Thus of the eight Ids in this study, each was unique in that none of the α-Ids reacted specifically with mAbs other than the complementary Id used for its induction.

The eight Ids used in this study were present in only low concentrations in individual mice immunized with Sindbis virus (<5 μg/ml). The rabbit α-Ids prepared to them did not evoke detectable anti-Sindbis virus antibodies when inoculated into mice (data not shown).

TABLE 1

PROPERTIES OF MONOCLONAL ANTIBODIES

				Reactivity with[c]					
mAb	IP[a]	IgG	Nt[b]	AR	v23	v18	v50	v49	HR
49	E2	G2a	+	+	+	+	±	−	+
50	E2	G2b	+	+	+	+	−	−	+
23	E2	G3	+	+	−	±	+	−	−
18	E2	G2a	+	+	−	−	+	−	−
51	E2	G2b	+	+	−	−	+	−	−
33	E1	G2b	+	+	+	+	+	+	+
K42	E1	G2a	−	+	+	+	+	+	+
53	C	G2a	−	+	+	+	+	+	+

[a] Protein immunoprecipitated by the mAb: glycoprotein E1, glycoprotein E2, or capsid protein C. The mAbs reactive with either glycoprotein also inhibit hemagglutination.

[b] +, mAb neutralizes the infectivity of Sindbis strain AR339; −, mAb is nonneutralizing.

[c] Reactivity of the mAb with different strains of Sindbis virus assayed by ELISA. v23 signifies that the strain was selected to be resistant to neutralization by mAb23, etc. v23, v18, and v50 were derived from strain AR339, HRSP is a laboratory strain derived from AR339, and v49 was derived from HRSP. mAb K42 also reacts with a second alphavirus, Semliki Forest virus, whereas the other mAbs do not.

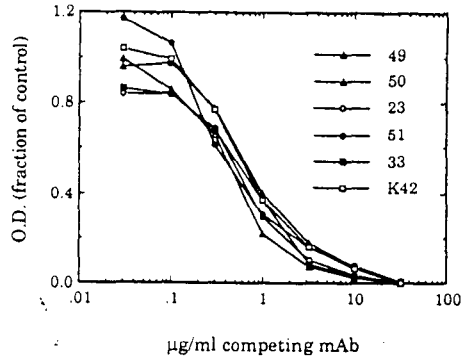

FIG. 1. Inhibition of binding of labeled mAb to purified α-Id antibody by unlabeled homologous mAb. 50 μl of 1.0 μg/ml purified α-Id antibody was affixed to polystyrene EIA plates. 50 μl of unlabeled homologous mAb (in 1% normal mouse serum) at the indicated concentrations were added for 60 min, followed by addition of 50 μl peroxidase-labeled idiotypic mAb sufficient to saturate the α-Id. After further incubation the OD was determined. Results are expressed as the fraction of OD developed relative to that developed in the presence in five heterologous noncompeting mAbs (see Table 2). Diluent only controls gave OD values from 0.84 to 0.98 of these averaged values.

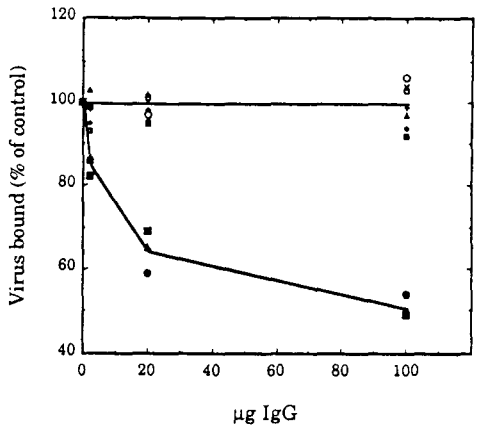

FIG. 2. Binding of ^{35}S-labeled Sindbis virus to chicken cells in the presence of affinity purified α-Id. Confluent monolayers of secondary chicken embryo fibroblasts in 96 well plates (5 × 10^4 cells/0.32 cm^2 well) were incubated for 60 min with heat-inactivated, affinity-purified α-Id in 100 μl PBS (Dulbecco and Vogt, 1954) on ice. Then 30 μl of ^{35}S-labeled Sindbis virions (AR339) (8457 cpm/μl, 8.3 × 10^7 PFU/μl) purified as described (Bell *et al.*, 1979), was added and the incubation continued for 2 hr. The monolayers were washed, dissolved in 0.5% SDS, and radioactivity bound determined by liquid scintillation counting. ■, α-Id49; ●, α-Id50; ▲, α-Id23; ◇, α-Id18; ⊡, α-Id33; □, α-IdK42; O, α-Id53; Δ, rabbit IgG Fc fragment (Boehringer-Mannheim); ×, rabbit IgG; +, rabbit anti-mouse IgG.

Binding of virus to cells

^{35}S-labeled Sindbis virus was purified and used to study binding of the virus to chicken embryo fibroblasts. The amount of virus bound as a function of input multiplicity was assayed and at apparent saturation, 5100 counts/min bound to 5 × 10^4 cells. Since the preparation had a specific infectivity of 9800 PFU/count/min, this corresponded to 1000 PFU bound/cell. The absolute specific infectivity of this preparation of virus was not determined. Previous studies of the binding of Sindbis virus AR339 and its derivatives to chicken cells by an electron microscopic technique (Birdwell and Strauss, 1974), or to a number of other cell lines using binding of radiolabeled virus (Smith and Tignor, 1980), gave results of about 10^5 particles bound per cell at apparent saturation, suggesting that the specific infectivity of the preparation used here may have been on the order of 1%.

We next assayed the ability of affinity purified α-Ids to block the binding of virus. Chicken cells were pretreated at 0°C with α-Id, and then near saturating amounts of labeled virus were added and incubation continued at 0°C. After washing the cell-bound label was determined. The ratios of virus bound in the presence of α-Id relative to control IgG are shown in Fig. 2. Three α-Ids, α-Id23, α-Id50, and α-Id49, all to neutralizing mAbs that react with glycoprotein E2, clearly inhibit virus binding to chicken cells, although only partially. The α-Id to mAb18, which is also a neutralizing antibody that reacts with E2, those to 33 and K42, which are reactive with glycoprotein E1, and that to 53, which is reactive with the capsid protein (Table 1), show insignificant virus blocking activity.

TABLE 2

SPECIFICITY OF ANTIIDIOTYPIC ANTIBODIES[a]

	Competing mAb					
α-Id	49	50	23	51	33	K42
	OD (fraction of control)					
49	0.00	0.82	0.99	1.03	1.06	1.09
50	0.85	0.00	1.00	1.01	1.04	1.11
23	0.81	0.79	0.00	1.16	1.04	1.20
51	0.99	0.95	1.05	0.00	0.97	1.05
33	0.85	0.88	1.08	1.02	0.00	1.17
K42	1.03	0.89	0.96	1.06	1.06	0.00

[a] Competition assays were performed as in Fig. 1, with competing mAb at 32 μg/ml in each case.

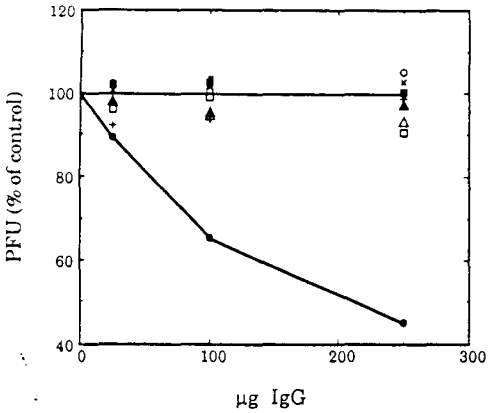

FIG. 3. Reduction in plaques upon adsorption of virus in the presence of protein A–Sepharose-purified IgG from α-Ids. Duplicate monolayers of secondary chicken embryo fibroblasts in 60-mm culture dishes were treated with 500 μl of α-Id in PBS at 50, 200, and 500 μg/ml. After incubation for 90 min at room temperature, 500 μl of PBS containing approximately 200 PFU of Sindbis virus was added and incubation continued for 60 min at room temperature. The inoculum was removed and the monolayers overlaid with Eagle medium containing 1% agarose. After overnight incubation at 37°C, the plaques were visualized by staining with neutral red. The results shown are the average of two independent experiments. ●, α-Id49; ❶, α-Id18; ○, α-Id23; □, α-Id33; ■, α-Id50; △, α-Id51; ▲, α-Id53; +, preimmune serum; ×, rabbit IgG Fc fragment.

Plaque reduction assay

We wished to develop a more convenient assay to examine the blocking of virus binding to cells. For this monolayers of secondary chicken fibroblasts were pretreated with α-Id for 90 min at room temperature. Then approximately 200 PFU of virus were added and the virus allowed to absorb for 60 min at room temperature in the presence of the α-Id, following which the cells were washed, overlayed with agarose, and plaques allowed to develop. Under the conditions used, it requires more than 2 hr to reach a plateau of virus uptake as measured by the number of plaques formed (data not shown). Thus the number of plaques formed would be expected to be sensitive to the concentration of available receptors on the cell.

Results from a plaque reduction experiment in which several protein A-purified IgGs from the α-Id antisera were tested are shown in Fig. 3. The blocking obtained with α-Id49 is comparable to that obtained with the affinity purified IgG in the saturation binding assay shown in Fig. 2. Under these conditions, however, α-Id23 and 50 fail to block. One possible source for the discrepancy is the different temperatures used in the two assays. If α-Id23 and α-Id50 have a low affinity for a receptor in comparison to α-Id49, they may be able to compete with the virus for the receptor at 0° but not at room temperature.

Binding of α-Id to the cell surface

We wished to determine if any α-Id would bind to the surface of uninfected cells, as would be expected if it were functioning as an antireceptor antibody. In preliminary experiments monolayers of chicken cells were treated with α-Id, then a fluorescently labeled goat anti-rabbit IgG was added, and the cells examined in a fluorescence microscope. Cells reacted with α-Id49 were clearly fluorescent whereas cells reacted with control sera and other α-Ids were not (data not shown).

To quantitate the extent of binding of the α-Id, chicken cells were removed from a culture plate with EDTA and treated with α-Id, fluorescently-labeled second antibody was added, and the cells were examined with a fluorescence-activated cell sorter. The results are shown in Fig. 4. α-Id49 clearly binds to the surface of uninfected chicken cells, and the population of chicken cells appears to be fairly uniform with respect to binding. There was no binding of α-Id33 or 51 above the preimmune level. The results with α-Id23 and 50 were intermediate.

Hamster cells

Since Sindbis virus has a wide host range, we wished to determine whether the putative antireceptor antibody identified above could recognize receptors on cells of a different origin. BHK-21 cells have been widely used for alphavirus studies and are efficiently infected by Sindbis virus, and are susceptible to a number of alphaviruses that, unlike Sindbis virus, grow poorly in avian cells (for example, Ross River virus; unpublished data). Plaque reduction assays in BHK cells using α-Id49 showed no demonstrable effect of this antibody upon Sindbis virus binding (Fig. 5), implying that the structure recognized by this antibody is not used as a receptor in these cells.

We also tested the ability of α-Id49, 23, 50, 33, and 51 to bind to the surface of BHK cells. There was no binding of any of these antibodies to BHK cells above the preimmune level (Fig. 4 and data not shown).

Immunoprecipitation of a chicken protein

Membrane fractions labeled with [^{35}S]Met were isolated from chicken fibroblasts and from BHK cells and screened with a number of the α-Ids for possible immunoprecipitation of specific proteins. No specific pro-

tein was precipitated by any α-Id from BHK cell fractions (data not shown). However, α-Id49, but no other α-Id, was found to specifically immunoprecipitate a protein of 63,000 Da from chicken cells (Fig. 6).

Sindbis virus variants

Variants of Sindbis virus have been isolated that are resistant to many of the mAbs used here (Table 1) (Stec *et al.*, 1986). We reasoned that if α-Id49 functioned as an antireceptor antibody because of molecular mimicry, then a variant resistant to mAb49, termed v49, might enter the cell through a receptor not bound by α-Id49. To examine this, a plaque reduction experiment was performed to test the ability of α-Id49 to block binding not only of the parental strain of Sindbis virus (AR339) but also of v49 and a number of other strains, including variants resistant to mAbs 23 and 50 (Fig. 7). v49 was insensitive to the presence of α-Id49, whereas the other four variants tested were all sensitive to the same extent.

DISCUSSION

Use of α-Ids to study the Sindbis virus receptor

The data presented here are consistent with the hypothesis that α-Id49 functions as an antireceptor antibody for a receptor that functions in chicken cells. It binds to the surface of chicken cells and blocks the binding of Sindbis virus by 50%. The fact that this antibody has no effect on Sindbis virus variant v49 makes it unlikely that it simply interferes with virus uptake in some nonspecific fashion. And the fact that it has no effect upon uptake of virus by BHK cells makes it unlikely that it interferes with virus uptake by interacting with the virus rather than the cell. α-Id49 specifically immunoprecipitates a protein of 63,000 Daltons from chicken cells, and this protein may function as a receptor for Sindbis virus in these cells.

Two of the other α-Ids tested, α-Id23 and α-Id50, appear to be low affinity antireceptor antibodies. They partially blocked virus binding at 0° but did not reduce the number of plaques formed upon adsorption of the virus at 20°. In fluorescence activated cell sorting experiments they bound to the surface of chicken cells at greater than preimmune levels, but not as avidly as α-Id49. Two other α-Ids made to anti-E2 mAb had no detectable antireceptor activity in the cells tested, nor did two α-Ids made to anti-E1 mAb.

Two previous studies have used different techniques in an attempt to identify a receptor for alphaviruses. Maassen and Terhorst (1981) found that a protein of M_r 90,000 could be specifically crosslinked to Sindbis glycoproteins upon binding of the virus to two lymphoblastoid cell lines. They suggested that this protein functioned as a receptor for Sindbis for these

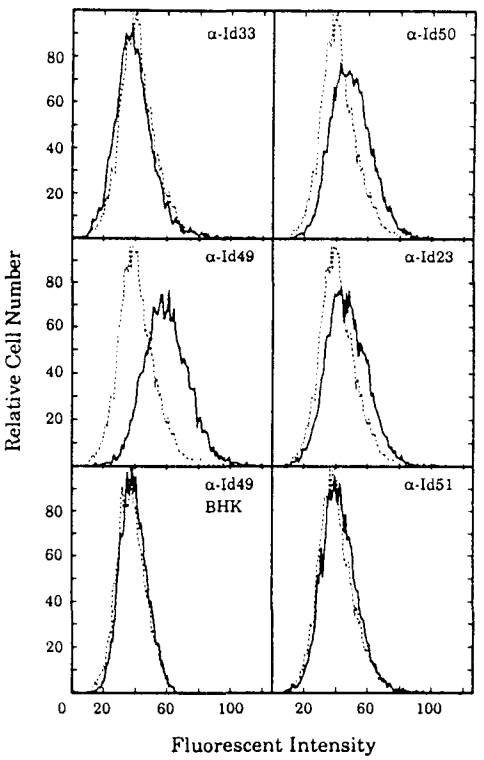

FIG. 4. Binding of α-Ids to the cell surface. Monolayers of chicken cells or BHK cells were washed with PBS lacking divalent cations and removed from the culture dish by treatment with 4 m*M* EDTA in PBS for 10 min at 37°. The cells were washed, treated with 100 μg/ml DNase I, and 10 μg of affinity purified α-Id added to 10^6 cells in a volume of 250 μl. After a 40-min incubation on ice, the cells were washed, a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG (ICN) was added in the same final volume, and the mixture incubated for an additional 30 min on ice. The cells were washed, resuspended in 1 ml, and filtered through a nylon screen (Tetko Instruments). 5 μl of 10 μg/ml propidium iodide (Sigma) was added and flow cytometric analysis performed, using a modified Ortho System 50H flow cytometer. An argon laser (Coherent) provided excitation at 488 nm (500-mW output power). Signals from dead cells were gated out electronically using a combination of red propidium iodide fluorescence and forward angle light scatter. Each histogram of fluorescein fluorescence intensity was based on signals from 10^4 live cells. Dashed lines indicate the distribution of cells labeled with preimmune serum, solid lines with the α-Id. All of the results shown are for chicken cells except for the panel marked BHK.

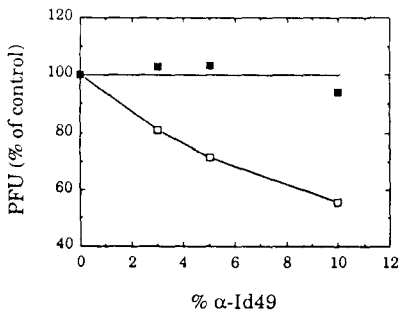

FIG. 5. α-Id49 does not cause a reduction in plaques in BHK cells. BHK or secondary chicken cell monolayers were treated with the indicated concentrations of unpurified α-Id49 antiserum, and a plaque reduction assay performed. ■, BHK cells, □, chicken cells.

two cell lines. The relationship of this protein to the 63,000 Da protein immunoprecipitated from chicken cell membranes by α-Id49 remains to be determined.

Helenius *et al.* (1978) reported that another alphavirus, Semliki Forest virus (SFV), could bind specifically to antigens of the major histocompatibility complex, the H-2 antigens of mice or the HLA antigens of humans, and they suggested that these MHC antigens functioned as receptors for SFV. It was subsequently reported by Oldstone *et al.* (1980) that SFV would infect a lymphoblastoid cell line, Daudi, which does not express HLA antigens and, therefore, that MHC antigens were not required for entry of SFV into a cell. In view of the findings that more than one receptor may be utilized by alphaviruses for entry into cells, it is possible that MHC antigens could serve as receptors on some cells and Daudi cells could express another receptor.

Multiple receptors used by alphaviruses

Because α-Id49 (or α-Id23 or 50) blocks virus binding by only 50%, it appears that there are at least two different receptors for Sindbis virus in chicken cells. Furthermore, the failure of the antibody to bind to or to block virus binding to BHK cells suggests that at least some of the receptors in BHK cells and chicken cells are distinguishable. The ability to use multiple receptors could be responsible in part for the very wide host range of the alphaviruses, which replicate in arthropods and in a wide range of vertebrates. In other cases in which protein receptors for animal viruses have been identified, only a single protein has been found to function as a receptor, and this appears to limit the host range of the virus.

The results with Sindbis virus variant v49, selected to be resistant to mAb49, also suggest that there are multiple receptors on chicken cells for the virus. This variant is able to infect chicken cells but virus uptake is not interfered with by α-Id49. We presume this variant either uses those wild-type receptors not blocked by

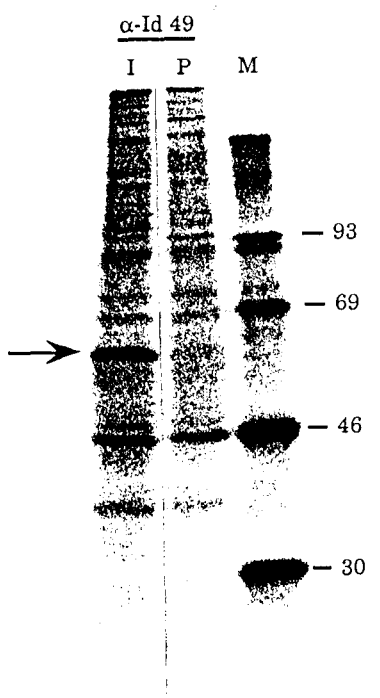

FIG. 6. Precipitation of a chicken protein by α-Id49. Secondary chicken embryo fibroblasts in 800-cm² roller bottles were labeled with 25 μCi/ml [^{35}S]Met (Amersham) for 12 hr. The cells were washed, removed from the bottle by treatment with 4 m*M* EDTA in PBS, and collected by centrifugation. The cell pellets were resuspended in 1 m*M* $NaHCO_3$ containing 10 m*M* EDTA and protease inhibitors (1 m*M* PMSF, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin), lysed by Dounce homogenization, and the nuclei removed by centrifugation for 5 min at 2000 *g*. A membrane extract was prepared by centrifugation for 1 hr at 115,000 *g* and resuspending the pellet at 4° in 3% Triton X-100 in 50 m*M* Tris–Cl, pH 7.5, 0.3 *M* NaCl, 200 μg/ml BSA, 4 m*M* EDTA, and protease inhibitors. Insoluble material was removed by centrifugation, and immunoprecipitation performed essentially as described (Hardy and Strauss, 1988), but using Affi-gel Protein A (BioRad) to collect the antigen-antibody complexes. Lane I: immunoprecipitation with α-Id49 serum. Lane P: immunoprecipitation with preimmune serum from the same rabbit. The 63,000 Da protein is indicated by the arrow. Lane M contains markers whose molecular size in kilodaltons is indicated.

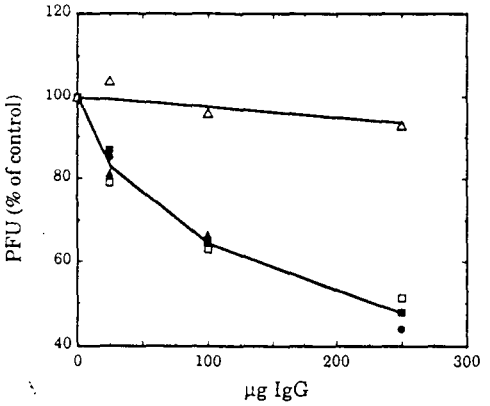

FIG. 7. Plaque reduction by α-Id49 for five different variants of Sindbis virus. The results shown are the average of two independent experiments. ■, strain AR339; ●, strain HR; ▲, strain v23; □, strain v50; △, strain v49.

α-Id49, or that change(s) in glycoprotein E2 of v49 allows the virus to bind to a new set of receptors. In this regard it is of interest that attempts to isolate a variant resistant to mAb49 from the AR339 parental strain of Sindbis virus were not successful (unpublished results). However, attempts using a derivative of AR339 referred to as HR, which differs from AR339 at several positions in both glycoproteins (Polo *et al.*, 1988; E. Strauss, A. Schmaljohn, D. Stec, and J. Strauss, in preparation), were successful. Evidently changes in glycoprotein E2 of Sindbis virus strain AR339 rendering it unreactive with mAb49 are lethal, but at least one such change in strain HR gives rise to the viable variant v49.

Previous studies have also indicated that multiple receptors may be used in the entry of Sindbis virus. Smith and Tignor (1980) found that a neurovirulent strain of Sindbis virus (AR86) and the avirulent prototype strain of Sindbis virus (AR339) did not interfere with one another for binding to a variety of cell types, and they suggested that these two strains of Sindbis virus utilize different receptors. Furthermore, there were approximately 25-fold more receptors for the AR86 strain than the AR339 strain of Sindbis virus on a neuroblastoma cell line (N18), suggesting that this might explain the much greater virulence of AR86 in mice. Interestingly, they also found that AR86 and Eastern equine encephalitis virus partially interfered with one another for binding to C6 rat astrocytoma cells, suggesting that these two different alphaviruses, both of which are neurovirulent, may use a common receptor for entry into cells. The lack of complete blocking suggested that more than one receptor is used by each virus strain and that only a portion of the receptors used were the same for the two viruses. These authors also found that binding of Sindbis virus to different cell lines was sensitive to treatment with pronase, suggesting that the virus receptor was a protein.

In another set of studies, Symington and Schlesinger (1975, 1977) isolated a variant of Sindbis virus that bound to and infected mouse plasmacytoma cells more efficiently than did the parental virus. They concluded that a change in the surface charge of the variant was important, and that evidently the variant could overcome an electrostatic barrier that prevented the parental Sindbis virus from attaching efficiently to plasmacytoma cells but not to chicken or BHK cells. It is unclear whether this phenomenon signifies that the receptors utilized in these different cell lines are different, or that plasmacytoma cells have a greater surface charge that leads to electrostatic repulsion.

The nature of the receptor

If the hypothesis that there are multiple receptors for Sindbis virus on the surface of cells is correct, it is unclear whether these receptors represent different forms of the same protein which are immunologically distinguishable, or different proteins that are closely related and contain the same epitope recognized by the virus as a receptor, or whether there might in fact be totally different receptors used by the virus. It is conceivable that during virus evolution it adapted to more than one receptor as it acquired a very wide host range. Isolation and characterization of the receptors will be required to resolve this question, and the possession of antireceptor antibodies makes it possible to directly isolate and characterize a virus receptor. We are in the process of isolating sufficient amounts of the chicken protein to allow its characterization. Of greater interest is the fact that the success of the approaches used here points the way for the isolation of immune reagents effective for other cell lines or other alphaviruses, which will allow characterization of receptors used by Sindbis virus in different cells or of receptors used by two different alphaviruses in the same cell, and we are attempting to isolate an antireceptor antibody for BHK cells in order to compare the chicken and BHK receptors. Full length clones of alphaviruses have been developed (Rice *et al.*, 1987; Davis *et al.*, 1989; R. J. Kuhn, H. G. M. Niesters, Z. Hong, and J. H. Strauss, in preparation) which make possible site specific mutagenesis or the construction of chimeric viruses that differ in their virulence in model systems

(Lustig *et al.*, 1988). Thus there is the potential to manipulate the virus in ways that change its interactions with receptors, and to correlate the distribution of receptors with virulence and tissue tropism. Finally, Sindbis virus has been proposed as a vector that allows efficient expression of foreign sequences in the cytoplasm of a broad range of cells (Xiong *et al.*, 1989). An understanding of virus–receptor interactions would make the virus potentially more useful as an expression vector.

ACKNOWLEDGMENTS

We are grateful to E. Rothenberg and E. Strauss for many stimulating discussions and for help in preparing the manuscript, and to R. Diamond for help with the FACS analysis. This work was supported by NIH Grant AI 20612 to J.H.S. Early portions of the α-Id work were supported by Office of Naval Research Contract N00014-84-K-0536 to A.L.S.

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Chapter 4

Mapping Neutralization Epitopes on Glycoproteins of Sindbis Virus

The chapter was prepared for submission

Introduction

Antibodies that bind to surface structures of a virion can interfere with the uptake and uncoating of a virus, a process called neutralization of infectivity. The neutralization epitope consists of the sequence or structure to which such a neutralizing antibody binds. In at least some cases an antibody neutralizes by binding to the structure on the virus that interacts with a receptor on the cell surface, thus directly blocking the virus from interacting with its receptor (Mandel, 1982). Various strategies have been followed to map the neutralization eptiopes of viruses. These include analysis of genetic variants that are resistant to an antibody (Diamond et al., 1985; Stec et al., 1986) and assay of the interaction of the antibody with peptides, either short polypeptides produced synthetically or by fragmentation of the protein or expressed as fusion proteins in a convenient system (Lerner, 1882).

Sindbis virus is an enveloped virus with two glycoproteins, E1 and E2, at the surface (Strauss and Strauss, 1986). Neutralization antibodies specific for both E1 and E2 have been isolated and studied (Schmaljohn et al., 1983). A study of variants resistant to many of these neutralizing antibodies has shown that variants in a domain of E2 from 180 to 220 are selected with many of the E2-specific antibodies (Strauss et al., 1991). We have also shown (Chapter 3) that antiidiotypic antibodies to some of these neutralizing antibodies function as antireceptor antibodies in chicken cells, suggesting that the neutralization epitopes defined by these antibodies function at least in part as the virus antireceptor that binds to the host cell receptor.

Bacteria expressing vector λgt11 have been well developed for isolating a particular gene by expressing a specific peptide. Because the site of insertion for foreign DNA in λgt11 is within the structural gene for β-galactosidase, foreign DNA sequences in this vector have the potential to be expressed as fusion proteins with β-galactosidase. Recombinant cDNA libraries constructed in λgt11 can be screened with antibody probes for

peptides produced by specific recombinant clones (Young and Davis, 1983). We have used this system in an attempt to define the neutralization epitopes of E2 and E1 in order to compare these epitopes with the results from antibody-resistant variants and to define more accurately the domain of the glycoproteins that appears to function in binding of the virus to receptors on the host cell.

Materials and Methods

Virus Growth and RNA Purification

Sindbis virus strain AR339 was obtained from A. Schmaljohn and was grown in monolayers of primary chicken embryo fibroblasts as previously described (Pierce et al., 1974). Virus was purified as described (Bell et al., 1979), disrupted with 0.5% SDS, and 49S genomic RNA was extracted using the phenol/chloroform method of Hsu *et al.* (1973). After two ethanol precipitations, RNA was resuspended in distilled H_2O and stored at -70°C.

Construction and Screening of the Phage Library

A λgt11 library containing short inserts of Sindbis cDNA was constructed by a modification of the procedure of Young and Davis (1983). cDNA synthesis was randomly primed with sonicated salmon testis DNA, using Sindbis virion RNA as template. [^{32}P]dCTP was included during cDNA synthesis. The cDNA product was flush-ended with the Klenow fragment of DNA polymerase I, methylated with *Eco*RI methyltransferase, phosphorylated *Eco*RI linkers (Collaborative Research) were added, and the modified cDNA was digested with an excess of *Eco*RI restriction enzyme. The cDNA was then fractionated on a Sephadex CL-6B column, and Sindbis cDNA fragments of 100-300 bp in size were pooled and ligated to dephosphorylated λgt11 arms (Promega). After *in vitro* packaging into phage heads (Stratagene), the percentage of phage containing Sindbis virus cDNA inserts was scored by plating phage on *E. coli* Y1090 in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactosidase (x-gal), followed by plaque-immunoreaction screening with mAbs. Phage plaques were grown for 6 hrs at 42°C, nitrocellulose disks (Schleicher & Schuell) soaked in 10 mM isopropyl thio-β-D-galactopyranoside were then placed on the top of the agar layer, and the plates were transferred to 37°C for 15 hrs. The filters then were lifted and washed successively in 10

mM Tris Cl pH 7.5 and 150 mM NaCl containing 5% nonfat milk (Carnation). The filters were incubated overnight at 4°C with monoclonal antibody (10 μg/ml in PBS containing 5% nonfat milk), washed, ^{125}I-conjugated protein G (0.5 μCi/ml in 5% nonfat milk) added, and the filters were incubated for at least 2 hr at room temperature. After washing and drying, the filters were exposed overnight at -80°C to Kodak-X-OMAT film. Immunoreactive phage were picked and rescreened until a uniformly reactive population was obtained.

DNA Purification and DNA Sequence Analysis

Positive λ DNAs were purified by a liquid medium lysate procedure (Young and Davis, 1983). The inserts were removed with *Eco*RI, subcloned into vector M13mp18, and sequenced by the dideoxy chain termination procedure (Sanger et al., 1977).

Results

Mapping the Location of Neutralization Epitopes

A library of randomly generated 100 to 300 base pair Sindbis cDNA fragments inserted into the *Eco*RI site in the lacZ gene of λgt11 was constructed. In this vector, translational open reading frames are expressed as part of a β-galactosidase fusion protein. With this procedure, we expected that immunoreactive phage would carry sufficiently small Sindbis virus cDNA inserts to enable precise mapping of the neutralizing epitopes on the Sindbis virus glycoprotein E1 and E2. We used a radioactive screening procedure in which ^{125}I-protein G was used as a second reagent to detect the presence of mAbs (all various IgGs) bound to immunoreactive phage clones on the nitrocellulose filters. Four positive phage clones were identified when mAb 23 was used to screen 10^6 plaques, designed λ23a, λ23b, λ23c and λ23d. These four phages were plaque purified and DNA prepared from each. The Sindbis inserts in each phage were subcloned into M13mp18 and sequenced by the dideoxy chain termination procedure. All four inserts contained overlapping sequences from the central region of glycoprotein E2, which contains a total of 423 residues.(Fig 1). The insert in λ23a comprised E2 residues 155-258, that in λ23b comprised residues 173-251, that in λ23c 145-223, and that in λ23d 169-220. Thus the domain from residues 173 to 220 is present in all four inserts, and the neutralizing epitope recognized by mAb 23 must lie within this region.

We also attempted to identify phage immunoreactive with four other neutralizing mAbs reactive with E2, namely mAbs 18, 50, 51, and 49, as well as phage immunoreactive with mAb 33, an mAb that is reactive with glycoprotein E1. In each case 10^6 plaques were screened. No positive plaques could be identified with any of these antibodies. We conclude that these antibodies probably react with conformational epitopes not present in the λgt11 library.

Discussion

The λgt11 system provides a rapid, specific, and sensitive strategy for the physical mapping on large viral genomes of the genes encoding proteins for which antibody reagents are available. Through the use of small Sindbis virus genomic inserts, portions of genes containing specific epitope sequences may be recognized by monoclonal antibodies for further biochemical or genetic manipulations. The limitation of the system is the fact that these protein domains are expressed as part of a fusion protein and thus may not fold in the same way as the native protein, and only antibodies that interact with contiguous linear domains of the proteins of interest may be reactive with phage plaques.

We examined six neutralizing monoclonal antibodies directed against Sindbis virus glycoproteins E1 and E2 for their ability to recognize short segments of the glycoprotein sequence expressed in λgt11. Only one of the mAbs tested, mAb 23, reacted with the λgt11 library. From the sequence of the inserts in four clones immunoreactive with mAb 23, we determined that this antibody was able to react with a single continuous region of the Sindbis glycoprotein E2, and that the neutralization epitope must lie within the 48 residues between amino acids 173 and 220. This region is hydrophilic, containing 25% charged residues, and has a glycosylation site at Asn-196, and thus is almost certainly exposed on the surface of the glycoprotein spike (Strauss and Strauss, 1986). Previous work mapping neutralization escape variants of mAb 23 as well as of other E2-specific mAbs, including mAb 49, have identified this same region as forming an important antigenic determinant (Strauss et al., 1991). In Chapter 3 were reported results that antiidiotypic antibody to mAb 49, and to a lesser extent antiidiotypic antibodies to mAbs 23 and 50, functioned as an antireceptor antibody in chicken cells, suggesting that this E2 domain might form part of the antireceptor on the virus spike that binds to the cellular receptor. This hypothesis is supported by the observation that two strains of Sindbis virus that differ only in having Gly or Arg at residue 172 of E2 differ in their ability to bind to neuroblastoma cells in culture and differ in their neurovirulence for mice (Tucker and Griffin, 1991).

The other four E2-specific neutralizing mAbs examined, 18, 49, 50 and 51, appeared to require conformations not present in the λgt11 library, and thus to react with discontinuous or distributed epitopes.

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Figure Legend

Fig. 1.The position within the Sindbis virus glycoprotein E2 of neutralization epitope sites were determined by dideoxy DNA sequencing. Immunoreactive phage inserts are represented in different color lines. The overlapping region is shown as box.

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      |   10      |   20      |   30      |   40      |   50      |   60
  1 SVIDDFTLTS PYLGTCSYCH HTVPCFSPVK  IEQVWDEADD NTIRIQTSAQ FGYDQSGAAS 60
 61 ANKYRYMSLK QDHTVKEGTM DDIKISTSGP  CRRLSYKGYF LLAKCPPGDS VTVSIVSSNS 120
121 ATSCTLARKI KPKFVGREKY DLPPVHGKKI  PCTVYDRLKE TTAGYITMHR PRPHAYTSYL 180
181 EESSGKVYAK PPSGKNITYE CKCGDYKTGT  VSTRTEITGC TAIKQCVAYK SDQTKWVFNS 240
241 PDLIRHDDHT AQGKLHLPFK LIPSTCMVPV  AHAPNVIHGF KHISLQLDTD HLTLLTTRRL 300
301 GANPEPTTEW IVGKTVRNFT VDRDGLEYIW  GNHEPVRVYA QESAPGDPHG WPHEIVQHYY 360
361 HRHPVYTILA VASATVAMMI GVTVAVLCAC  KARRECLTPY ALAPNAVIPT SLALLCCVRS 420
421 ANA                                                              423
      |   10      |   20      |   30      |   40      |   50      |   60
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totally 423 amino acid residuces

λ23a
λ23b
λ23c
λ23d