TARGETING PROTEINS FOR UBIQUITINATION AND DEGRADATION IN THE TREATMENT OF HUMAN DISEASE

Thesis by

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ABSTRACT

Protein degradation is one of the tactics employed by the cell for irreversibly inactivating proteins. In eukaryotes, ATP-dependent protein degradation in the cytoplasm and nucleus is carried out by the 26S proteasome. Most proteins are targeted to the 26S proteasome by covalent attachment of a multiubiquitin chain. A key component of the enzyme cascade that results in attachment of the multiubiquitin chain to the target or labile protein is the ubiquitin ligase that controls the specificity of the ubiquitination reaction. Defects in ubiquitin-dependent proteolysis have been shown to result in a variety of human diseases, including cancer, neurodegenerative diseases, and metabolic disorders.

The SCF (Skp1-Cullin-F-box-Hrt1) complex is a heteromeric ubiquitin ligase that multiubiquitinates proteins important for signal transduction and cell cycle progression. A technology was developed known as Protac (Proteolysis Targeting Chimeric Molecule), that acts as a bridge, bringing together the SCF ubiquitin ligase with a protein target, resulting in its ubiquitination and degradation. The Protac contains a peptide moiety at one end that is recognized by SCF that is chemically linked to the binding partner or ligand of the target protein. The first demonstration of the efficacy of Protac technology was the successful recruitment, ubiquitination, and degradation of the protein Methionine Aminopeptidase-2 (MetAP-2) through a covalent interaction between MetAP-2 and Protac. Subsequently, we demonstrated that Protacs could effectively ubiquitinate and degrade cancer-promoting proteins (estrogen and androgen receptors) through non-covalent interactions *in vitro* and in cells. Finally, cell-permeable Protacs can also promote the degradation of proteins in cells. Biologically, this work signifies the amazing versatility and flexibility of the ubiquitin-proteasome system. Technologically, this work represents

the development of a novel “chemical genetics” approach to selectively target proteins for degradation. Practically, this work is “Proof of Concept” that exploiting the cell’s natural proteolytic machinery is a potential avenue for the treatment of human disease.

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NOMENCLATURE

$SCF^{\beta\text{-}TRCP}$. Skp1-Cullin-Fbox

Protac. Proteolysis Targeting Chimeric Molecule

E2. Estradiol

DHT. Dihydroxytestosterone

Methionine aminopeptidase-2. MetAP-2

ER. Estrogen receptor

AR. Androgen receptor

Chapter I

INTRODUCTION

Ubiquitin (Ub)-dependent proteolysis of key regulatory proteins impacts various cellular processes such as cell cycle progression, transcription, antigen presentation, receptor endocytosis, fate determination, and signal transduction (1, 2). With so many cellular pathways affected, it is therefore not surprising that derangements in the Ub-proteolytic pathway contribute to the etiology of several diseases. In this review, we will summarize our current understanding of how Ub-dependent degradation takes place in the cell, and the various steps at which an impairment of the normal pathway contributes to the diseased state. We will conclude by summarizing the different ways we, as well as others, are utilizing the cellular degradation machinery to develop therapeutic strategies to treat human diseases.

BACKGROUND

The Ubiquitin System

Ubiquitin is one of the most conserved proteins in eukaryotes. It is a small 76-amino acid protein that is conjugated to other proteins through an energy-dependent enzymatic pathway (1, 3). Conjugation is initiated by the activation of Ub by the Ub-activating enzyme, E1, which forms a high-energy Ub-thiol ester bond in the presence of ATP. It then transfers the activated Ub to a Ub-conjugating enzyme, E2, forming an E2-thiol ester bond. Finally, ubiquitin is transferred to a target substrate protein through an isopeptide linkage between the conserved C-terminal glycine residue of ubiquitin and the ε-amino group of the lysine residue of the substrate. In many cases, the transfer of ubiquitin

from an E2 to a target protein requires the involvement of a ubiquitin ligase, E3, which is discussed in the next section. Sequential conjugation of the internal lysine residue of ubiquitin to a C-terminal glycine residue of a new ubiquitin molecule results in formation of a polyubiquitin chain, which targets proteins for degradation by the 26S proteasome (Fig. 1) (4, 5).

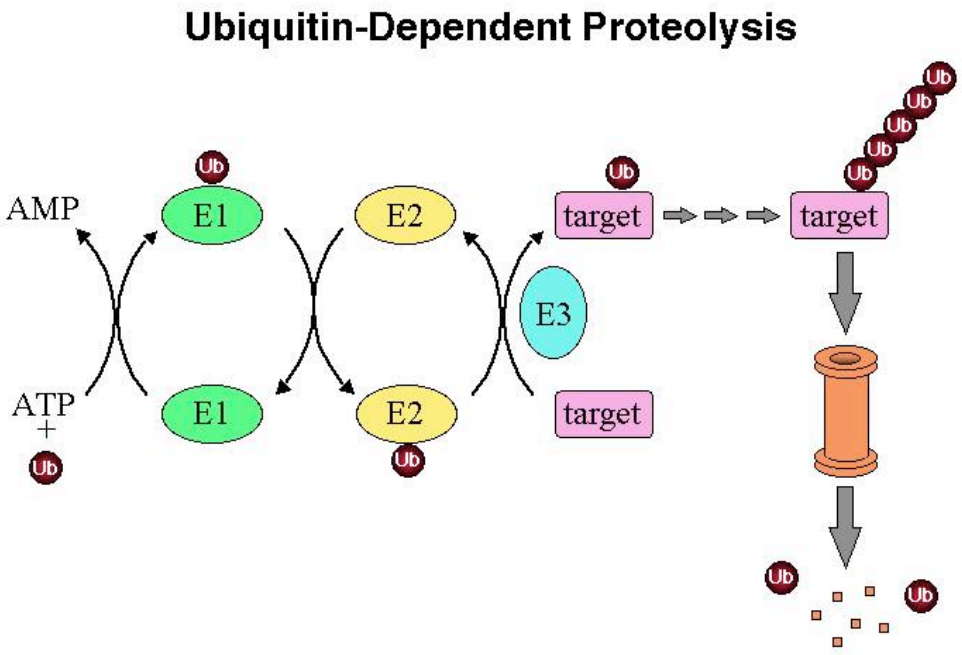

Fig. 1

Ubiquitin Ligases

The E3 ubiquitin ligases cooperate with E2 ubiquitin conjugating enzymes to assemble multi-ubiquitin chains on substrate proteins. Generally, ubiquitin ligases, whether monomeric, or multimeric complexes, contain domains that recognize substrate proteins specifically, as well as domains that interact with E2 enzymes, thereby promoting ubiquitin

transfer from the E2 to the substrate. Based on sequence homology and catalytic mechanisms, all known ubiquitin ligases can be divided into two major classes: the catalytic HECT domain E3s, and the adaptor RING finger based E3s (4, 6). Variants of the latter class, the PHD domain containing E3s and the U box E3s are only now beginning to be defined (7-9).

The HECT domain proteins are found in all eukaryotes and are defined by a 350 amino acid C-terminal HECT homology domain (Homologous to E6-AP C-Terminus), originally identified in E6-AP (10). E6-AP is the cellular ubiquitin ligase recruited by the human papilloma virus E6 oncoprotein to induce degradation of the p53 tumor suppressor. Multiple HECT domain proteins have been identified, including RSP5, UFD4, and TOM1 in *S. cerevisiae*; Pub1 in *S.pombe*, and NEDD4 in humans. The large N-terminal domains allow the ligases to bind substrates and the C-terminal HECT domains serve to directly transfer ubiquitin to the substrates through an E1-E2-E3 ubiquitin thiol ester cascade. Thus this class of ligases functions catalytically (6, 11).

The identification of RING finger domains in SCF (Skp1-Cullin-F box) and other non-HECT domain ubiquitin ligases suggested that these domains function to ubiquitinate proteins. The RING domain is a zinc-binding motif that is defined by an octet of cysteine and histidine residues in a consensus sequence CX2CX (9-39)CX(1-3)HX(2-3)C/HX2CX(4-48)CX2C (4, 6, 12). RING finger E3s play prominent roles in diverse cellular processes, including cell cycle progression, signaling, transcription, apoptosis, proliferation and DNA repair.

The SCF subfamily of E3s was originally discovered and studied in budding yeast *S. cerevisiae.* The SCF complex consists of at least four subunits: CUL1/CDC53,

HRT1/RBX1/ROC1, SKP1 and an F-box protein (4, 13, 14). The known SCF subunits are conserved throughout evolution. The Cullin and the RING finger protein HRT1 form a catalytic core of E3s that binds to and activates E2 CDC34 or UBCH5 (15, 16). HRT1 is a small 121 amino acid RING finger protein that binds directly to Cullins and CDC34, likely tethering E2 to the CH domain, which is confirmed by the crystal structure (17). SKP1 binds to the CDC53/HRT1 core and mediates recruitment of various F-box adapter proteins, which contain a 45 amino acid motif called an F-box and bind to substrates through protein-protein interaction domains, thus conferring substrate specificity to this family of E3s. Multiple F-box proteins have been shown to bind SKP1. There are over 400 F-box proteins currently in the database, with 20 F-box proteins in S. cerevisiae, over 100 in *C. elegans*, and over 50 described so far in mammals (18).

Many proteins have been demonstrated to be substrates of SCF ubiquitin ligase (4). These proteins participate in a variety of cellular functions, including regulation of cyclin dependent kinase activity, activation of transcription, signal transduction and DNA replication. In all cases of SCF Ub-ligases working in conjunction with the Cdc34 E2 enzyme, phosphorylation of the substrates is required for recognition by the F-box proteins. For SCF^{CDC4}, each of the known substrates, SIC1 (14, 19), CDC6 (20), and GCN4 (21, 22), FAR1(23), must be phosphorylated before they can be recognized by the WD40 domain of CDC4 and ubiquitinated. Another WD40-repeat protein, β-TRCP, forms a complex with SCF and recognizes a specific phosphoserine motif found in its substrates IκBα and β-catenin (24-26). This mechanism allows for differential and temporal regulation of SCF substrate stability in the presence of fully active SCF complexes.

SCF might serve as a prototype for other modular Cullin-based ubiquitin ligases. There are at least five other human cullins, which do not bind SKP1. Human CUL2 binds to the Von Hippel-Lindau gene product (VHL) through Elongin B and Elongin C (which shares homology with the N-terminal region of SKP1) to form a complex (CUL2-VBC) that displays ubiquitin ligase activity (27). VHL down-regulates hypoxia-inducible mRNAs presumably by controlling proteolysis of hypoxia regulated transcription factors HIF1α and HIF2α (28, 29). Human CUL3 was recently shown to ubiquitinate Cyclin E and control S phase in mammalian cells (30). Human CUL4 associates with UV-damaged DNA binding protein and appears to play a role in DNA repair (31, 32).

Another prominent RING finger containing E3 is the Anaphase Promoting Complex (APC/Cyclosome), which is required for metaphase to anaphase transition and mitotic exit (33). APC is a multisubunit ubiquitin ligase that consists of at least 12 proteins, including Cullin family member APC2 and a RING-H2 protein APC11 (34, 35). As with SCF, APC associates with WD repeat-containing adapter proteins CDC20/fizzy and CDH1/HCT1/Fizzy-related that activate APC toward specific substrates (36).

A subclass of ubiquitin ligases is the Plant homeodomain (PHD) or Leukemia associated-protein (LAP) domain containing E3s, which have eight conserved metal binding ligands (C4HC3) with similar spacing to ring finger ligases, to which they are closely related structurally (37). The PHD/LAP domain is found in more than 400 eukaryotic proteins, many of which are involved in regulating gene expression (38). PHD domains have been reported to have a variety of functions, including protein-protein interaction and binding to DNA. Recently, it was shown that the MEKK1 PHD domain has E3 ubiquitin ligase activity, suggesting at least in some cases, these proteins may

behave as E3s (7). Naturally occurring mutations have been identified in several diseases, such as ING1 in head and neck squamous cell carcinomas (39), AIRE in autoimmune polygladular syndrome, type I (40), and MLL and CBP in myeloid leukemias (41). Furthermore, genes encoding PHD proteins have been identified in deletion regions of several continuous gene deletion syndromes such as Williams syndrome (42). The occurence of mutations in PHD domains suggests that the PHD domain E3 activity could play an important role in human disease (43).

Recently, a sequence-profile analysis demonstrated that the U box is a derived version of the RING-finger domain that lacks the metal-chelating residues but is likely to function similarly to the RING-finger in mediating ubiquitin-conjugation of protein substrates. Interestingly, the signature cysteines of the RING finger are not conserved in the U box. Multiple alignment of the U box with selection of RING fingers shows that, except for the loss of the hallmark cysteines and a histidine, the U box retains the same pattern of amino acid residue conservation. Thus, the U box, like the RING finger are likely to activate ubiquitination and multi-ubiquitination by facilitating the interaction between E2 proteins and their substrates (9).

Protein Degradation in Human Disease

Diseases arising due to impairment of ubiquitin ligases

Cervical cancer

Among the best-characterized associations between cancer and the ubiquitin ligase pathway is that of cancer of the uterine cervix. This is the third most common cancer diagnosed in women. While the precise cause of cervical cancer remains uncertain, the disease is strongly associated with infections by the oncogenic forms of HPV, types 16 and

18. The E6 and E7 proteins of these high risk strains are often detected in cervical cancers (44). In these same carcinomas, the levels of tumor suppressor p53 are very low, suggesting a link between E6 and p53. In fact, the p53 protein forms a ternary complex with high risk HPV E6 oncoprotein E6-16 or –18 and the ubiquitin ligase E6-AP (10). E6-AP can interact with E6 in the absence of p53, but can only interact with p53 in the presence of E6. The E6/E6-AP complex promotes ubiquitination of p53, resulting in degradation by the 26S proteasome. The strong correlation between different polymorphisms of p53 to E6-mediated destruction and the prevalence of cervical carcinoma in women further supports the link between p53 degradation by the ubiquitin pathway and malignant transformation. The tumor suppressive effects of p53 are most likely exerted through its apoptotic function or checkpoint activity such that accelerated degradation promotes malignant transformation.

SCF complex and cancer

The invariant subunits of SCF are the E2-interacting subunits Hrt1/ROC1, Cdc53/Cul1, and the F-box interacting protein Skp1. The subunit that varies is the F-box containing protein that confers substrate specificity. Thus the specific form of the SCF ligase is denoted by a superscript. Besides SCF^{Cdc4}, SCF^{Skp2} is another form of the ligase whose substrate is the cyclin-dependent kinase (cdk) inhibitor, p27Kip1. Recently, a cdk subunit known as Cks1 was found to direct ubiquitin-mediated proteolysis of the CDK-bound p27Kip1 by SCF^{Skp2} (45). The p27 protein plays a major role in maintaining differentiated mammalian cells in a quiescent state by negatively regulating the activities of cyclin-dependent kinases required for initiation of DNA synthesis and inhibiting G1 to S transition (46). The p27 protein is unstable and expressed at low levels in many cancers

(47). In many cases, decreased p27 expression is associated with a worse prognosis (48, 49). Further support for the role of Skp2 in regulation of p27 levels *in vivo*, mouse embryonic stem cells lacking the Skp2 gene express elevated levels of p27 (50).

Regulation of G1 to S transition in the mammalian cell cycle occurs through the activation of cyclin dependent kinases (Cdks) 2, 4, and 6. The cyclin that regulates Cdk2, Cyclin E, is targeted for ubiquitin-mediated proteolysis by an SCF ubiquitin ligase that contains the human homologue of yeast Cdc4, which is an F-box protein containing WD40 repeats. Recently, the F box protein Cdc4 was found to be mutated in breast cancer cells that express high levels of cyclin E (45). In one breast cancer cell line, a tandem duplication consisting of a direct repeats of exons 8 and 9 separated by 11 base pairs of intronic sequence. This mutation was predicted to result in chain termination, eliminating the last four WD40 repeats, rendering the truncated Cdc4 nonfunctional. Interestingly, aberrant hCdc4 mRNA levels, loss of hCdc4 protein, and loss of heterozygosity in these cell lines were also observed (45).

Targets for $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase include the transcription factor β-catenin and IκB, the inhibitor of the transcription factor NF-κB (25, 26, 51). $SCF^{\beta\text{-}TRCP}$ complexes recruit phosphorylated β-catenin and IκB for ubiquitination by the E2 enzyme Cdc34 as well as ubch5. β-catenin functions as a transcription factor in the Wnt/wingless signaling pathway (52). The Wnt/wingless family of secreted proteins act as inducers of axis formation and organogenesis in embryonic patterning pathways of many developing tissues. In the absence of a Wnt/wingless signal, β-catenin is rapidly phosphorylated by the glycogen synthase 3-β (GSK-3β) kinase in a reaction that requires axin and the

adenomatous polyposis coli (APC) tumor suppressor protein. Phosphorylated β-catenin is targeted for ubiquitination by the SCF$^{\beta\text{-TRCP}}$ complex and degraded by the proteasome (53). In the presence of a Wnt/wingless signal, β-catenin is not phosphorylated and ubiquitinated. The levels of transcriptionally active β-catenin rise, which results in activation of β-catenin-regulated gene expression. Mutations of β-catenin phosphorylation sites, as well as APC mutations also block ubiquitination and degradation of β-catenin and often result in inappropriate expression of β-catenin-regulated genes and in cancer (52, 54). Recent work has suggested that a complex of casein kinase I and axin induces β-catenin phosphorylation at serine 45 that initiates the degradation cascade and serves as a molecular switch for the Wnt pathway (55).

SCF$^{\beta\text{-TRCP}}$ complex and immune modulation

The SCF$^{\beta\text{-TRCP}}$ complex also participates in activation of transcription factor NF-κB by targeting the inhibitor IκB for ubiquitination and degradation by the proteasome, as well as being implicated in processing of the p105 precursor. Extracellular signals mediated by cytokines, such as TNFα activate phosphorylation of IκB by the IκB kinases (56). Phosphorylated IκBα is targeted for ubiquitination by the SCF$^{\beta\text{-TRCP}}$ complex and degraded by the proteasome, resulting in nuclear translocation of NF-κB and activation of expression of NF-κB-regulated genes. More recent work demonstrated that the signal transducer in the NF-κB pathway, TRAF6, a ring domain protein, functions together with a ubiquitin conjugating enzyme Ubc13/Uev1A to catalyze the synthesis of unique polyubiquitin chains linked through lysine 63 (K63) of ubiquitin. Inhibition of this polyubiquitin chain synthesis

prevents the activation of IKK by TRAF6 (57). This suggests a new role for ubiquitination in immune regulation.

von Hippel-Lindau (VHL) disease

The VHL syndrome is an autosomal dominant familial cancer syndrome that predisposes affected individuals to tumors, including cerebellar hemangioblastomas, hemangiomas, renal cell carcinomas, retinal angiomas, and pheochromocytomas (58). The VHL tumor suppressor gene is located on chromosome 3p25.5 and is mutated in the majority of sporadic clear cell renal carcinomas in addition to tumors associated with the VHL syndrome. The VHL protein resides in the cytoplasm but can translocate to the nucleus that is Ran and energy-dependent. VHL forms stable, multimeric complexes that contains Elongin B, Elongin C, Cul2, and Rbx1 (59). The knowledge that Elongin C resembles Skp1 adaptor protein of SCF and VHL associates with the cullin family member Cul2, suggested that the VHL/Elongin/Cul2 complex might function similarly to SCF in yeast. The crystal structure of VHL bound to Elongin B and C showed that the region of VHL that binds to Elongin C loosely resembles an F-box and that Elongin C resembles Skp1 (29). Mutations found in VHL disease clustered in two regions that are critical for complex formation and potentially substrate recognition. Furthermore, anti-VHL immunoprecipitates contained ubiquitin ligase activity, indicating that VHL was associated with ubiquitination. It is now known that VHL ubiquitinates HIF transcription factor, which regulates hypoxia inducible genes and is degraded in the presence of oxygen (59). Furthermore, VHL binds to a short HIF-derived peptide on a conserved proline residue only when the peptide is hydroxylated. Since proline hydroxylation requires molecular

oxygen and Fe2+ , this modification most likely plays a critical role in oxygen sensing in mammalian cells (59).

Clear cell renal carcinoma cells lack a functional VHL protein and exhibit a diverse array of phenotypes including cell cycle defects (60), overexpression of TGF (61), defects in endoplasmic reticulum associated degradation of misfolded proteins (62), and defects in assembly of extracellular matrix (63). One interesting feature of VHL tumors is their high vascularity, which is thought to result from deregulation by VHL of hypoxia-inducible genes including the angiogenesis-promoting factor vascular endothelial growth factor (VEGF)(64, 65). The expression of VEGF and other hypoxia-regulated genes are repressed under normoxic growth conditions in cells, but are strongly induced under hypoxic conditions and constitutively expressed in cells lacking functional VHL.

Cbl ubiquitin ligase and cancer

Cbl is an adapter protein that contains both SH2 and RING-HC finger domains. Cbl functions as a monomeric ubiquitin ligase that recognizes phosphorylated tyrosine on receptor tyrosine kinases (RPTKs) through its SH2 domain and negatively regulates signaling by promoting active receptor ubiquitination and degradation (66-70). Oncogenic versions of Cbl containing deletions or point mutations in the C-terminal tyrosine kinase binding domain resulted in transformation. Another oncogenic form of c-Cbl, termed 70Z-Cbl, exhibits a 17-amino acid deletion that removes C381, the first cysteine residue of the RING finger, and most of the linker domain between TKB and the RING finger. These mutants lack E3 activity, suggesting that they act as dominant-negative proteins competing with wild-type c-Cbl for tyrosine-phosphorylated tyrosine kinases abrogating the negative-regulatory effect of c-Cbl in cell signaling (68).

Angelman's Syndrome

Angelman's syndrome is a rare neurological disorder characterized by mental retardation, seizures, absence of speech, excessive laughter, and abnormal gait. The affected gene is the E3 ubiquitin ligase E6-AP. It is thought that the syndrome results from aberrant accumulation of substrates of E6-AP (71). Although the targets are unknown, studies have suggested a role for E6-AP in brain development. Studies also show that mutation of the E6-AP ubiquitin ligase reduces nuclear inclusions and accelerate polyglutamine-induced neurodegeneration (72).

Parkinson's disease

One of the most common forms of familial Parkinson's disease is the autosomal recessive juvenile Parkinsonism (AR-JP). AR-JP is characterized by selective dopaminergic neural cell death and the absence of the Lewy body, a cytoplasmic inclusion body consisting of aggregates of abnormally accumulated proteins (73).

AR-JP was first described by Yamamura and colleagues (74). The gene was mapped to chromosome 6q25.2-q27 and contains 12 exons with a molecular mass of 52 kDa. This gene is highly conserved throughout evolution, including *Drosophila, c. elegans,* and mammals. The N-terminus of Parkin demonstrates a ubiquitin-like domain, while the C-terminal half region of Parkin has two RING-finger motifs. Parkin is thought to act as a ubiquitin ligase through association with the ubiquitin-conjugating enzyme UbcH7. Mutations in Parkin in AR-JP patients demonstrate decreased ubiquitin ligase activity (75). Recent findings suggest that accumulation of proteins that are substrates of Parkin causes selective neuronal cell death without formation of Lewy bodies.

Further link between Parkinson's Disease and the ubiquitin system has been suggested in the studies of two gene products, UCH-LI (ubiquitin carboxyl-terminal hydrolase) and □-synuclein, whose mutations cause autosomal dominant familial Parkinson's Disease (76, 77). UCH-L1 is thought to regenerate ubiquitin by both cleaving polyubiquitin chains and releasing ubiquitin from small adducts such as glutathione and cellular amines (78). □-Synuclein, one of the major components of Lewy bodies (79) is degraded by the 26S Proteasome (80), suggesting that it is modified by ubiquitin, and mutations in Parkin may extend the half-life of the protein.

Other neurological disorders including Huntington's disease and spinocerebellar ataxias have been thought to also result from mutant proteins that aggregate in intranuclear accumulation bodies that fail to be degraded due to expanded CAG/polyglutamine repeats (81, 82). In prion diseases, pathology results from accumulation of a conformationally altered prion protein, which forms aggregates (83). Thus, it may be postulated that prionogenic and amyloidogenic proteins escape correct protease and chaperone responses of the cellular quality control program.

Diseases arising due to impairment of substrates of ubiquitin ligases

Alzheimer's disease and the ubiquitin system

In many neurodegenerative disorders, such as Alzheimer's disease, inclusions containing ubiquitinated proteins have been identified in the brain, suggesting a role for ubiquitin-dependent proteolysis of neuronal proteins. Prevention of ubiquitination inhibited the neurotoxic effects of □-amyloid. In the central nervous system, proteasome-mediated protein degradation plays a major role in the breakdown of proteins damaged by oxidative stress or other insults, including glucose and oxygen deprivation. Inclusions

containing ubiquitinated proteins are commonly found in many neurogenerative disorders (84). Protein aggregation can also directly impair the function of the ubiquitin-proteasome system (85). Since Δ-amyloid itself could cause protein ubiquitination, and Δ-amyloid toxicity can be blocked by inhibiting protein ubiquitination or proteasome activity, new prophylactic and therapeutic avenues for treatment of neurodegenerative disease are being developed. Pharmacological inhibition of ubiquitination or proteasome-mediated degradation of ubiquitinated proteins may prevent, alleviate, or inhibit the progression of chronic neurogenerative diseases such as Alzheimer's disease.

Cystic fibrosis and ubiquitination

Cystic fibrosis (CF) is the most common genetic disorder and is characterized by severe bronchopulmonary infections and pancreatic insufficiency. The gene associated with CF encodes a transmembrane protein (CFTR) which is a chloride ion channel normally localized to the plasma membrane of epithelial cells. Because it is a large protein, most of the wild type protein is degraded from the ER by the ubiquitin system and only a small fraction reach the cell surface. In CF, mutations in the protein interfere with the folding of the protein, the most common of which is ΔF508. Although it is functional, the ΔF508 protein is completely retained in the ER, from which it is polyubiquitinated and degraded by the proteasome (86, 87). The efficient degradation of the mutant protein leads to a lack of expression of ΔF508 CFTR at the cell surface, resulting in CF (88). In this disease, a number of therapeutic strategies are possible, including transient transfection of airway epithelial cells with intact CFTR; modification of the post-transcriptional quality control mechanisms: activation of residual membrane-associated CFTR; activation of second

messenger pathways; activation of alternative CF channels in the luminal membrane; and inhibition of EnaC by amiloride.(88)

Wilson's disease

Wilson's disease is an autosomal recessive inherited disorder of copper metabolism that leads to neuronal degeneration and hepatic cirrhosis. The Wilson protein is a copper-transporting P-type ATPase that when mutated, becomes trapped in the ER and is presumed to be the molecular basis of disease (89). Like the CFTR ΔF508 mutant protein, the Wilson protein is rapidly degraded.

Liddle syndrome

In contrast to CFTR and Wilson's protein, stabilization of a normally degraded protein is the basis of Liddle syndrome. This disease is an autosomal dominant form of hypertension characterized by early onset hypertension and hypokalemia, and suppression of plasma renin activity and aldosterone. The Liddle syndrome results from a deletion of the proline rich region in the C-terminal β and γ subchannel (ENaC), which leads to constitutive activation of the protein. ENaC normally has a short half-life because the β and γ chains are rapidly ubiquitinated. NEDD4 is a HECT domain protein that binds to the proline rich motif of ENaC through a highly conserved WW domain. Mutations in the β or γ subunits result in stabilization (90), leading to reabsorption of sodium and water and development of hypertension.

Diabetes mellitus

The hereditary disorder diabetes mellitus has been shown to be caused by missense mutations in the insulin receptor (IR). Various mutations in the IR gene have been

reported in individuals with severe insulin resistance (91). Several of these mutations result in decreased numbers of IRs at the cell surface, either as a consequence of impaired processing of the mutant proreceptors and accumulation in the ER or increased degradation of the mutant proreceptors. Recent studies have demonstrated that the degradation of mutant IR is preceded by a cleavage of the misfolded proreceptor, resulting in the accumulation of two proteins, 120kDa and 80kDa, which associate with the IR (91). The precise role of these interacting proteins has not been determined.

α1-antitrypsin Z

α1-antitrypsin (α1-AT) is the most common cause of infantile liver disease and also causes adult-type emphysema. Most α1-AT-deficient individuals are protected from liver damage by rapid degradation of the mutant α1-AT in the ER, however, some of these patients who develop severe liver disease, there is decreased degradation of α1-AT resulting in ER accumulation and hepatotoxicity (93). The protein appears to first translocate in a retrograde manner into the cytosol, since components of the ubiquitin proteasome pathway have not been shown to exist in the ER. As in the cases of α1-antitrypsin, CFTR, and Wilson's protein, the ubiquitin ligases that attach the ubiquitin onto these proteins have not been identified.

Cytolytic T cell response

The ubiquitin-proteasome system plays an important role in cellular immunity. Peptides from foreign antigens are presented as MHC class I molecules to elicit a cytolytic T cell response (94). In cells infected with viruses, viral antigens are targeted for degradation by the ubiquitin-proteasome pathway. The display of viral peptides on MCH class I molecules

is a critical component of host defense. Therefore, the ability to avoid the CTL response to viral infections is an effective way for a virus to escape immune surveillance in the host (95). An example is the Epstein-Barr virus (EBV) in which the Epstein-Barr nuclear antigen (EBNA) is expressed in latently infected B lymphocytes where it persists in healthy individuals. It is the only viral protein regularly detected in EBV-associated malignancies such as nasopharyngeal carcinomas and lymphomas, suggesting that escape from immune surveillance might promote transformation (95). The human cytomegalovirus (CMV) avoids immune surveillance by down-regulating MCH class I. Finally HIV utilizes an ER-associated proteasomal degradation pathway to induce the down-regulation of its CD4 receptor in infected cells (96). In the case of HIV, unlike EBV and CMV, the E3 that ubiquitinates CD4 is known. Proteasomal degradation of CD4 occurs through a ternary complex between CD4, the viral Vpu protein, and the WD protein β-TRCP, which recruits the Skp1 adaptor protein of the SCF complex (98). Vpu is an 81-amino acid membrane phosphoprotein that interacts with CD4. In HIV infected cells, expression of the viral envelope glycoprotein precursor gp160 results in the formation of stable CD4-gp160 complexes that are trapped in the ER. The VPU expressed in the ER then associates with the CD4 cytoplasmic tail and recruits β-TRCP to the ER membrane. β-TRCP, in turn, recruits Skp1, resulting in CD4 ubiquitination by the E3 ligase, SCF.

Potential Therapeutic Applications

Given the fact that all mammalian cells have ubiquitin ligases and proteasomes, selectively targeting proteins for ubiquitination and degradation is a potential avenue for drug development. Moreover, since there is recent information about the crystal structure

of ubiquitin ligases and their substrates, one could imagine using low molecular weight compounds to stabilize or degrade proteins to treat a variety of diseases. Several approaches have been considered, including gene therapy to selectively target proteins for ubiquitin-dependent proteolysis. Another approach has been to use chimeric molecules to recruit disease-promoting proteins to E3s for ubiquitination and subsequent degradation. Tyrosine kinase inhibitors also promote protein degradation through chaperones. We will discuss these approaches and their potential therapeutic applications.

Selective degradation of non-SCF targets by chimeric F box proteins

Recently it was shown that one could successfully engineer a substrate receptor of an F-box protein to direct the degradation of otherwise stable cellular proteins in yeast and in mammalian cells. The yeast F box-containing Cdc4 protein normally targets the phosphorylated cyclin-dependent kinase inhibitor Sic1 for degradation. In order to target the tumor suppressor Retinoblastoma (Rb) to the SCF machinery in yeast, derivatives of Cdc4 were fused to the E7 protein encoded by the human papillomavirus type 16. Using the N-terminal 35 residues of E7 (E7N), which contains a conserved LXCXE Rb binding motif, hybrid F box proteins were produced containing the F box/WD-40 repeat domains of Cdc4 sufficient to bind Skp1 and the HPV-16 E7N fused in frame (99). In yeast, Rb was shown to be degraded in cells expressing the fusion protein. Similar hybrid proteins were engineered using the mammalian F box containing β-TRCP fused to HPV-16 E7N. This fusion protein targeted Rb for degradation and inhibited RB-induced growth arrest in human osteosarcoma SAOS-2 cells. These results suggest that the mammalian and yeast SCF machinery could be harnessed to degrade targets that are not normally substrates of SCF. Such an approach is a potentially useful tool to evaluate whether a protein is a target

for drug intervention or to knock out cellular proteins to study their functions. As a therapeutic modality, there are limitations as with other gene therapy approaches, such as efficiency of transduction, duration of expression, and targeting to the right cells.

Ubiquitination and tyrosine kinase inhibitors

Recently, tyrosine kinase inhibitors (TKIs) have been found to not only inhibit tyrosine phosphorylation, but also enhance ubiquitination and degradation of the receptor tyrosine kinase, ErbB-2. ErbB-2/HER-2 is associated with aggressive tumors, including breast cancer. Therapy to target the oncoprotein is currently being used to treat cancer patients. A potent, irreversible TKI, CI-1033 was found to alkylate a cysteine residue specific to ErbB receptors. The pathway stimulated by TKIs appears to be chaperone mediated, and is common to the heat shock protein 90 (Hsp90) antagonist geldanamycin through a stress-induced mechanism (100). More recently, geldanamycin dimers and hybrid compounds containing geldanamycin linked to estradiol or testosterone have been shown to induce degradation of the estrogen receptor or androgen receptor, respectively, in cancer cells (101, 102). These results suggest that selectively targeting chaperoned oncogene products for destruction is an alternative strategy to treat human diseases such as cancer.

Chimeric molecules to target proteins for ubiquitination and degradation

To circumvent the problem of transducing cells at high efficiency, we sought to deliberately target a protein to the SCF complex by developing a chimeric compound, known as a Proteolysis Targeting Chimeric Molecule (Protac).

As proof of concept, we first tested whether the Protac could recruit methionine aminopeptidase-2 (MetAP-2) to the $SCF^{\beta\text{-}TRCP}$ for ubiquitination and degradation *in vitro*

(CHAPTER II). A Protac was synthesized that contained at one end, the minimal 10 amino acid phosphopeptide sequence of IκB that is recognized by the F-box protein β-TRCP (26) and at the other end, the MetAP-2 binding compound, ovalicin. MetAP-2 binds to ovalicin covalently. We demonstrated that the Protac could recruit the MetAP-2 to the $SCF^{\beta\text{-}TRCP}$ complex resulting in ubiquitination. Addition of Protac also resulted in degradation of MetAP-2 in *xenopus* extracts (103).

To determine whether Protac could be generalized to other ubiquitin ligases, we performed ubiquitination assays with Cbl (CHAPTER III). Cbl is a monomeric ubiquitin ligase that attaches ubiquitin to signaling molecules and receptor tyrosine kinases resulting in proteolysis. We generated a Protac that consisted of ovalicin and the zap70 phosphopeptide, which binds Cbl (101). We demonstrated that Protac promotes ubiquitination of MetAP-2 by Cbl *in vitro*. These results suggest that Protac can be generalized to other ubiquitin ligases.

Finally, to test whether Protacs could recruit a protein to the SCF for ubiquitination and degradation of a different target that interacts through non-covalent interactions, we targeted the estrogen receptor (ER) (CHAPTER IV). We demonstrated that not only can the ER be targeted to the SCF for ubiquitination, but the ubiquitinated ER is degraded by the 26S proteasome *in vitro*.

Concluding remarks and future directions

The purpose of this thesis project is to establish a completely novel approach to treating diseases by utilizing the normal function of ubiquitin ligases in cells and targeting disease-promoting proteins for degradation. If cell permeable Protacs prove to increase

turnover and degrade proteins in cells, this would lead to potential therapeutic applications in patients with cancer and other diseases. Future work will focus on testing Protacs in animal models of diseases, including prostate and breast cancer. Ultimately, our goal is to translate this strategy into clinical trials in patients with a variety of tumors and human diseases.

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Chapter II

Protacs: Chimeric Molecules That Target Proteins to the SCF Complex for Ubiquitination and Degradation

The data presented in this chapter were generated in collaboration with Kyung B. Kim, Akiko Kumagai, Frank Mercurio, and Craig M. Crews (Published in PNAS 98: 8554-8559, 2001; Appendix).

Introduction

Degradation of cellular proteins is required for normal maintenance of cellular function, including proliferation, differentiation, and cell death. One of the major pathways to regulate proteins post-translationally is ubiquitin-dependent proteolysis. Ubiquitination occurs through the activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3), which act sequentially to catalyze the attachment of ubiquitin to lysine residues of substrate proteins (1). The E3s confer specificity to ubiquitination reactions by binding directly to substrate. Although the exact number of E3s can not be determined with certainty from sequence data, there are probably >100 distinct F-box containing E3s encoded within the human genome (2). One particular class of E3s, the heterotetrameric SCF complexes, consists of Skp1, a Cullin family member, the RING-H2 protein Hrt1 (also known as Roc1 or Rbx1), and an F box-protein (3). These components are conserved from yeast to mammals. The mammalian F-box protein, β-TRCP/E3RS, has been shown to bind IκBα, a negative regulator of NFκB (26). The $SCF^{\beta\text{-}TRCP}$ complex promotes the ubiquitination and subsequent degradation of IκBα, which results in activation of NFκB during the inflammatory response (3).

The recruitment of I□B□ to SCF$^{□\text{-TRCP}}$ is mediated by a ten-amino-acid peptide within I□B□, DRHDSGLDSM (4, 5). In response to diverse inflammatory signals, I□B□ kinase (IKK) phosphorylates this motif on both serines, which triggers binding of IkBa to □-TRCP. Because it is a well-defined ligand for a specific ubiquitin ligase, we sought to take advantage of this phosphopeptide to target an unrelated protein to SCF$^{□\text{-TRCP}}$ for ubiquitination and degradation. Previous reports have indicated that dimerization and oligomerization of proteins using synthetic ligands can be used to regulate signaling pathways that are ligand-dependent (6).

As proof of concept, we tested the ability of the I□B□ phosphopeptide to target methionine aminopeptidase-2 (MetAP-2) to SCF$^{□\text{-TRCP}}$. MetAP-2 catalyzes the cleavage of N-terminal methionine from nascent polypeptides (7). MetAP-2 appears to be the primary target of the potent angiogenesis inhibitors fumagillin and ovalicin (8, 9). Both of these compounds inhibit MetAP-2 by covalently binding histidine-231 in the active site. The consequent reduction in MetAP-2 activity is thought to block endothelial cell proliferation by causing p53-dependent arrest in G1 phase of the cell cycle (10). Importantly, MetAP-2 is not known to be ubiquitinated or a substrate for any SCF complex.

To determine whether MetAP-2 could artificially be targeted to SCF$^{□\text{-TRCP}}$, we synthesized a chimeric molecule (Protac-1; for Proteolysis-Targeting Chimeric molecule #1) that contained both the I□B□ phosphopeptide and ovalicin. We hypothesized that the phosphopeptide moiety would bind □-TRCP and the ovalicin moiety would bind MetAP-2, thereby recruiting MetAP-2 to SCF$^{□\text{-TRCP}}$ for ubiquitination (Fig. 1). In this paper, we report that Protac-1 indeed binds MetAP-2 to SCF$^{□\text{-TRCP}}$, and thereby promotes MetAP-2

ubiquitination and degradation. Demonstration that Protac-1 mediates the ubiquitination and degradation of a foreign substrate by SCF provides a basis to begin testing Protacs *in vivo* in addition to other targets known to promote disease.

Results

MetAP-2 specifically binds Protac-1 *in vitro*

The I□B□-ovalicin chimera, Protac-1, was synthesized as described in Materials and Methods. To demonstrate that purified MetAP-2 bound Protac-1, we incubated MetAP-2 (18mM) with increasing concentrations of Protac-1 (Fig. 2A). A Western blot analysis was performed with anti-MetAP-2 anti-serum. At 10 mM and 10 mM Protac-1, we observed two bands; the lower band represents unbound MetAP-2, and the upper band represents MetAP-2 bound to Protac-1. Addition of Protac-1 at higher concentrations did not increase the yield of MetAP-2–Protac-1 complexes, suggesting that only a fraction of the MetAP-2 molecules were active and able to bind Protac-1. Combining MetAP-2 with either free I□B□ phosphopeptide or free ovalicin did not yield the doublet observed with Protac-1. We also tested the specificity of MetAP-2 interaction with Protac-1 *in vitro*. Free ovalicin, but not free I□B□ phosphopeptide, inhibited the formation of the MetAP-2–Protac-1 complex (Fig. 2B). Therefore, our results demonstrate that MetAP-2 was specifically conjugated to the ovalicin moiety of Protac-1 in a concentration-dependent manner.

Protac-1 recruits MetAP-2 to SCF

Prior to testing the activity of Protac-1, we first adapted a previously described approach to isolate and assay $SCF^{\beta\text{-}TRCP}$ complexes *in vitro* (11). Lysates from 293T cells

transfected with plasmids that encoded β-TRCP and Cul-1 proteins tagged with the FLAG epitope at the N-terminus were immunoprecipitated with anti-FLAG antibody conjugated resin. Immunoblot analysis confirmed that all components of $SCF^{\beta\text{-}TRCP}$ were present in the anti-FLAG immunoprecipitate, including Skp1, Hrt1, and the transfected ^{FLAG}Cul-1 and ^{FLAG}β-TRCP (data not shown). Furthermore, control experiments confirmed that these immunoprecipitates promoted ubiquitination of IKK-phosphorylated GST- IκBα in a manner that was inhibited by the IκBα phosphopeptide and Protac-1 (data not shown; (7)). To determine whether Protac-1 could recruit MetAP-2 to $SCF^{\beta\text{-}TRCP}$, we first incubated MetAP-2 (18 mM) with Protac-1 (100 mM) for 45 minutes at room temperature. Following isolation of $SCF^{\beta\text{-}TRCP}$ complexes, the anti-FLAG beads were supplemented with the MetAP-2–Protac-1 mixture and rotated at room temperature for one hour. The beads and supernatant were then evaluated by Western blot analysis for the presence of MetAP-2. Anti-FLAG beads coated with $SCF^{\beta\text{-}TRCP}$, but not control beads preincubated with untransfected 293T cell lysates, specifically retained a fraction of the MetAP-2–Protac-1 complex and not the unliganded MetAP-2 (Fig.3). These results demonstrate that Protac-1 specifically recruited MetAP-2 to $SCF^{\beta\text{-}TRCP}$.

Protac-1 mediates the ubiquitination of MetAP-2

Since Protac-1 was able to tether MetAP-2 to $SCF^{\beta\text{-}TRCP}$, we next asked whether MetAP-2 could be ubiquitinated. To test this possibility, we supplemented anti-FLAG beads coated with $SCF^{\beta\text{-}TRCP}$ with ATP, MetAP-2–Protac-1, plus purified E1, E2 (human Cdc34), and ubiquitin. Following incubation, Western blot analysis was performed with anti-MetAP-2 antiserum. This experiment was repeated with two different preparations of

purified MetAP-2; one which contained primarily a 47 kDa autocatalyzed cleavage product (Fig. 4A) and the other which contained full length 67 kDa protein (Fig. 4B). In both cases, MetAP-2–Protac-1 was extensively modified in the presence of $SCF^{\beta\text{-}TRCP}$-coated beads, but not control beads (mock). Substitution of the methyl ubiquitin, which acts as a chain-terminator of polyubiquitination, collapsed the high molecular weight forms of modified MetAP-2 to a series of 2-3 bands migrating directly above unmodified MetAP-2–Protac-1 complex (Fig. 4C, compare lanes 2 and 3 with lane 4), confirming that MetAP-2 was ubiquitinated by $SCF^{\beta\text{-}TRCP}$.

We next tested whether MetAP-2 ubiquitination was dependent on Protac-1. As shown in Fig. 4C, MetAP-2 was not ubiquitinated in the absence of either $SCF^{\beta\text{-}TRCP}$ (lane 1) or Protac-1 (lanes 5 or 6; for some experiments, methyl ubiquitin was used in place of ubiquitin to simplify detection of ubiquitin conjugates). Moreover, unlinked ovalicin (50 μM) plus IκBα peptide (50 μM) were not able to substitute for the ovalicin–IκBα peptide conjugate (lane 7). Protac-1-dependent ubiquitination of MetAP-2 was specific, in that it was readily competed by free IκBα phosphopeptide (Fig. 4D, lane 3 versus lane 2). In contrast, free ovalicin did not compete (Fig. 4D, lane 4), presumably because it was unable to displace Protac-1 that was previously covalently linked to MetAP-2. Taken together, these observations indicate that Protac-1 specifically elicited ubiquitination of MetAP-2 by $SCF^{\beta\text{-}TRCP}$, and that successful targeting required that the two components of Protac-1 be bound together as a chimeric molecule.

MetAP-2–Protac-1 is degraded in Xenopus egg extracts

The experiments described above demonstrated that MetAP-2 was ubiquitinated in a Protac-1-dependent manner by highly purified $SCF^{\beta\text{-}TRCP}$. However, key issues are whether Protac-1 can specifically activate MetAP-2 degradation, and whether targeted degradation can be achieved by endogenous ubiquitin/proteasome pathway components at typical intracellular concentrations. To address these questions, we preincubated MetAP-2 with Protac-1 to allow the complexes to form, and then added the mixture to *Xenopus* egg extract supplemented with constitutively active IKK (IKK-EE) (12), okadaic acid, and ovalicin. The addition of IKK-EE and okadaic acid was intended to sustain phosphorylation of the IκBα peptide moiety of Protac-1, whereas ovalicin was added to prevent the further linkage of Protac-1 to MetAP-2. Remarkably, MetAP-2–Protac-1 complex (top band) but not MetAP-2 alone (bottom band) was mostly degraded by 30 minutes (Fig. 5). Degradation of MetAP-2–Protac-1 was attenuated in extracts supplemented with the proteasome inhibitors LLnL or epoxomicin (13), but not by other protease inhibitors (chymotrypsin, pepstatin, and leupeptin) added to the reaction. Moreover, addition of both IKK-EE and okadaic acid was required for optimal degradation of MetAP-2–Protac-1. We have similarly seen specific turnover of the MetAP-2–Protac-1 complex, but not free MetAP-2, in 3 independent experiments. Since the IκBα phosphopeptide does not have lysines and the ovalicin does not have free amino groups, it is unlikely that Protac itself serves as a target for ubiquitin-dependent proteolysis. Taken together, these results suggest that Protac-1 targeted MetAP-2 for degradation via the

proteasome. MetAP-2 turnover appeared to be very specific, in that maximal degradation required agents predicted to sustain phosphorylation of the I□B□ peptide.

Discussion

Since degradation of ubiquitinated proteins occurs rapidly in cells, we reasoned that ubiquitin-dependent proteolysis might provide effective means to modulate the phenotype of normal and diseased cells. Thus, we sought to develop a method to target proteins, at will, to the ubiquitin/proteasome pathway. The linchpin of the strategy described here is the development of chimeric molecules, referred to as Protacs, that link a desired target protein to a ubiquitin ligase. The method of ligand-regulated activation and termination of signaling pathways with synthetic ligands has been previously reported (6). As a target protein for a "proof of principle" experiment, we chose MetAP-2, which covalently binds the angiogenesis inhibitor, ovalicin. For the ubiquitin ligase we chose the $SCF^{\beta\text{-}TRCP}$ complex, for reasons described in more detail below. Although MetAP-2 has 36 lysines, it has not been reported to be an unstable protein *in vivo*, and it was not clear whether it would serve as a substrate for $SCF^{\beta\text{-}TRCP}$, no less be degraded by the ubiquitin/proteasome pathway (14). Our results suggest that Protac-1 can in fact recruit MetAP-2 to $SCF^{\beta\text{-}TRCP}$ for ubiquitination. In addition, we report that Protac-1 specifies degradation of MetAP-2 by the endogenous ubiquitin/proteasome pathway in *Xenopus* egg extracts.

We selected $SCF^{\beta\text{-}TRCP}$ as a candidate ubiquitin ligase for the development of Protac technology for two reasons. First, the apparent constitutive activity of SCF complexes enables a general strategy to manipulate normal or diseased cells. Second, the mechanism underlying substrate selection is well-understood for $SCF^{\beta\text{-}TRCP}$. Pioneering work by Ben-Neriah and colleagues demonstrated that a 10 amino acid internal phosphopeptide mediates

ubiquitination and degradation of I□B□ (4, 5). Subsequently, it was established that b-TRCP is the receptor that links this phosphopeptide to the ubiquitin/proteasome pathway (26, 115). Furthermore, while our work was underway, Zhou, Howley, and colleagues demonstrated that engineered SCF complexes can be used to target heterologous proteins for destruction (16). Zhou et al. fused the human papillomavirus E7 protein to the F-box proteins Cdc4 and b-TRCP to create chimeras, which assemble to form hybrid $SCF^{Cdc4\text{-}E7}$ and $SCF^{□\text{-}TRCP\text{-}E7}$ complexes. E7 binds tightly to Retinoblastoma protein (Rb), and the F box protein-E7 chimeras stimulate turnover of Rb via the SCF pathway in both yeast and mammalian cells. A limitation of the F-box fusion approach is that it depends upon gene transfer of the chimeric F-box protein, and thus its use is limited to transgenic organisms, and its potential use as a therapeutic strategy awaits the development of safe and reliable gene therapy protocols.

We propose that Protacs may be useful research tools for manipulating the phenotype of cells through the targeted elimination of specific proteins, or useful therapeutic agents for targeting the elimination of disease-promoting proteins. An obstacle to realizing these goals, however, is that the phosphopeptide-containing Protac-1 described here is unlikely to penetrate cells. For future applications, Protacs will need to be modified to enhance delivery to cells. For example, attachment of the tat peptide (17) may promote transduction of Protac-1 into cells, or a □-TRCP-binding peptide that is derived from the HIV Vpu protein (18, 19), and is phosphorylated by constitutively active Casein Kinase II, may allow delivery of an unphosphorylated Protac to cells. The ultimate goal is to identify

small molecules that can substitute for the E3-targeting activity of the I□B□ phosphopeptide.

We envision the potential to develop an entire suite of Protac compounds (Fig. II-6). Several approaches have been developed for identifying small molecules that bind to any target protein of interest (20-22). Candidates emerging from such screens would serve as platforms for the production of new Protacs, regardless of the topology of their interaction with the target protein. Moreover, many ubiquitin ligases have been discovered over the past few years, and with the completion of the human genome sequence, more ubiquitin ligases will undoubtedly follow. Further understanding of the substrate specificity of these ubiquitin ligases will provide insights into ways that they can be exploited for the development of novel Protacs.

Experimental Procedure

Synthesis of I□B□-ovalicin Protac

Ovalicin (1.4 mmol) was dissolved in 10 mL of methanol at 0 ^{o}C and $NaBH_4$ (3.0 mmol) was added slowly. After 30 min stirring, methanol was removed under reduced pressure and the resulting crude product was purified by flash column chromatography to yield ovalicinol (1.15 mmol, 82%). To the ovalicinol, Fmoc-Gly was coupled to give Fmoc-Gly-ovalicinol. Specifically, dimethylformamide (DMF) (28 μL) was added to dichloromethane solution (30 mL) containing Fmoc-Gly-OH (3.56 mmol) and oxalyl chloride (7.12 mmol) at 0^{o}C. After 3 hour stirring at room temperature, dichloromethane was removed under nitrogen atmosphere. The resulting solid residue was redissolved in dichloromethane (10 mL) and was combined with ovalicinol (0.6 mmol) and dimethylamino pyridine (DMAP) (4.7 mmol) in dichloromethane (30 mL) at 0 ^{o}C. The

reaction mixture was stirred for 2 hours at room temperature. After dichloromethane was removed under reduced pressure, the resulting residue was flash chromatographed to provide Fmoc-Gly-ovalicinol (0.39 mmol, 65 %). Next, Fmoc-Gly-ovalicinol (0.09 mmol) was treated with 20 % piperidine in DMF (2 mL) at room temperature for 10 minutes and DMF was removed under high vacuum. The resulting solid was redissolved in 2 mL of dimethylsulfoxide (DMSO) and disuccinimidyl suberate (DSS) (0.9 mmol) was added at room temperature. After overnight stirring, DMSO was removed under high vacuum and the resulting crude product was flash chromatographed to give monosuccinimidyl suberate-Gly-ovalicinol (0.06 mmol, 68 %). Monosuccinimidyl suberate-Gly-ovalicinol (12 μmol) in DMSO (0.6 mL) was added to DMSO solution (1 mL) containing I□B□ peptide (3.67 μmol) and DMAP (11 μmol). After 20 min stirring at room temperature, the coupling reaction was completed, which was confirmed by Kaiser test. DMSO was removed under high vacuum and the resulting crude product was repeatedly washed with dichloromethane and methanol to remove excess monosuccinimidyl suberate-Gly-ovalicinol to give the final product (I□B□ peptide-suberate-Gly-ovalicinol) (5.8 mg, 2.59 μmol, 70 %). The final product was characterized by electrospray (ES) mass spectrometry. ES-MS $(M+H)^+$ for ovalicinol-Gly-suberate-I□B□ peptide:DMAP, 2,231.56. All other intermediates were characterized by 500 MHz ^{1}H NMR spectroscopy.

MetAP-2–Protac-1 coupling assay

MetAP-2 (9 mM) was incubated with increasing concentrations of Protac-1 (dissolved in water) at room temperature for 45 minutes. Reactions were supplemented with SDS loading dye, fractionated on a 10% SDS-polyacrylamide gel, transferred onto a

nitrocellulose membrane and immunoblotted with rabbit polyclonal anti-MetAP-2 antiserum (Zymed, Inc.). ECL was performed using Amersham detection reagents.

Tissue culture and transfections

293T cells were cultured in DMEM with 10% fetal bovine serum (Gibco), Penicillin (100 units/ml)/Streptomycin (100 μg/ml), and L-glutamine (2 mM). Cells were split 1:5 prior to the day of transfection and transiently transfected with 40 μg of plasmid. Cells were 60% confluent in 100 mm dishes on the day of transfection. DNA (20 μg of pFLAG-Cul1[RDB1347] and 20 μg of pFLAG-β-TRCP[RDB1189]) by calcium phosphate precipitation. Cells were harvested 30 hours following transfection. Five micrograms of pGL-1, a plasmid containing the CMV promoter linked to the green fluorescent protein (GFP) cDNA was co-transfected into cells at the same time to assess transfection efficiency. In all experiments, greater than 80% of the cells were GFP–positive at the time of harvest.

Immunoprecipitation and ubiquitination assays

293T cell pellets were lysed with 200 μl of lysis buffer (TrisCl 25 mM, pH 7.5, NaCl 150 mM, Triton-X100 0.1%, and NaF 5 mM, EGTA 0.05 mM and PMSF 1 mM). Pellets from cells transfected with vector, pFLAG-β-TRCP or pFLAG-Cul-1 were vortexed for 10 seconds, then incubated on ice for 15 minutes. Following centrifugation at 13,000 rpm for 5 minutes at 4^{o}C, the supernatant was added to 20 μl of FLAG M2 beads (Sigma, Inc.), which were washed with lysis buffer three times prior to immunoprecipitation. Lysates were incubated with the beads on a rotator for two hours at 4^{o}C, followed by one

wash with Hepes buffer 25 mM, pH 7.4, Triton-X100 0.01%, and NaCl 150 mM (Buffer A) and one wash with the same buffer without Triton-X100 (Buffer B). For binding assays (Fig. 3), 50% (10 µl) of the 9 µM MetAP-2/50 µM Protac-1 mixture was loaded as input; the other 50% added to beads. Following addition of ligand, the beads were rotated at room temperature for 1 hour. The beads were washed once each with buffers A and B. Following centrifugation, half of the bead and supernatant fractions, representing bound and unbound, respectively, were evaluated by western blotting as described in '*MetAP-2–Protac-1 coupling assay*'. For ubiquitination reactions, 4 µl of 18 µM MetAP-2, 4 µl of 100 µM Protac-1, 0.5 ml of 0.1 µg/µl purified mouse E1, 1 ml 0.5 µg/µl human Cdc34 E2, and 1ml 25 mM ATP were added to 20 µl (packed volume) of washed FLAG-M2 beads. For competition experiments, the phosphopeptide (100 µM final) or ovalicin (100 µM final) was added simultaneously with the Protac-1. Reactions were incubated for one hour at 30^{o}C in a thermomixer (Eppendorf) with constant mixing. SDS-PAGE loading dye was added to terminate reactions, which were evaluated by western blotting as described in

MetAP-2–Protac-1 coupling assay

The experiments performed herein employed two different preparations of MetAP-2. One preparation consisted primarily of a 47 kDa fragment that was generated either by a contaminating protease, or slow autoproteolysis. The second preparation consisted almost entirely of full length 67 kDa MetAP-2. Essentially identical results were obtained with both preparations.

Degradation experiments with *Xenopus* extracts

Extracts from unfertilized *Xenopus laevis* eggs were prepared the day of the experiment. The MetAP-2-Protac-1 mixture (4 µl of 9 µM MetAP-2 plus 50 µM Protac-1) or MetAP-2 alone was added to 10 µl of extract in addition to ovalicin (10 µM final), IKK-EE (0.4 µg) and okadaic acid (10 µM final). LLnL (50 µM final), epoxomicin (10 µM final), or DMSO vehicle were added to the indicated concentrations to inhibit degradation by the proteasome. The protease inhibitors, chymostatin, pepstatin, and leupeptin (15 mg/ml final concentration), were also added to the extracts. Reactions were incubated for the indicated timepoints at room temperature and terminated by adding 50 µl of SDS-PAGE loading dye. Samples were evaluated by western blotting as described above.

Acknowledgements

We would like to thank the members of the Deshaies lab for their helpful suggestions and Zhen-Quiang Pan for providing reagents and valuable input on the reconstitution of $SCF^{\beta\text{-}TRCP}$ activity. This work was supported by UCLA Jonsson Comprehensive Cancer Center, CapCURE (KMS, RJD, and CMC), the Gates Grubstake Fund (RJD), and the Howard Hughes Medical Institute (RJD); KMS is a Scholar of the Leukemia and Lymphoma Society of America.

Figures

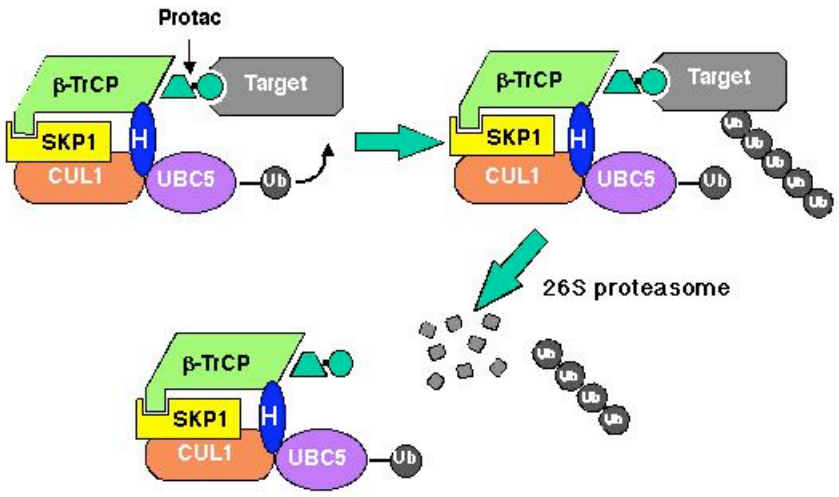


Figure II-1. Protac-1 targets MetAP-2 to SCF. Protac-1 is a chimeric molecule that consists of a phosphopeptide moiety and a small molecule moiety that interacts with the protein target (A). The synthesis scheme for Protac-1 is shown (B; see Materials and Methods).

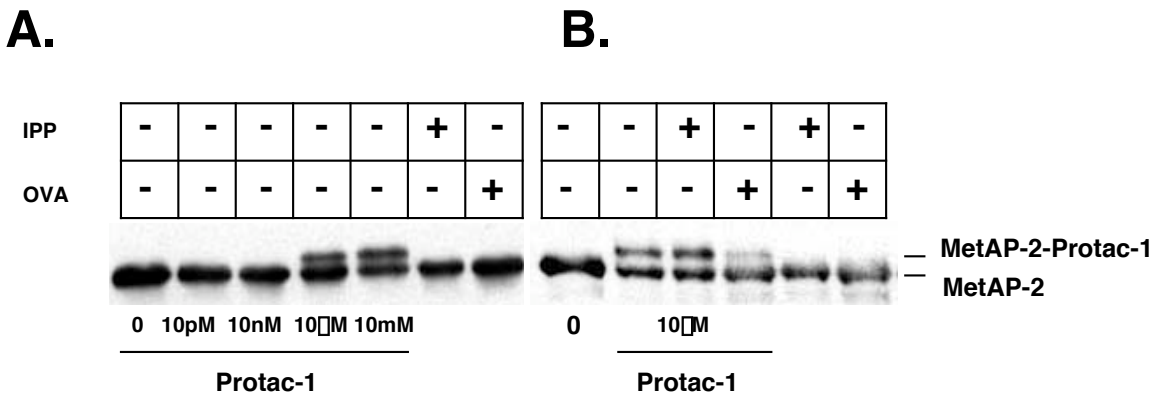


Figure II- 2. MetAP-2 binds Protac specifically and in a concentration-dependent manner.

(A): MetAP-2 (9 mM) was incubated with increasing concentrations of Protac-1 at room temperature for 45 minutes. The last two lanes depict MetAP-2 that was incubated with either free IκBα phosphopeptide = IPP (50 μM) or free ovalicin = OVA (50 μM), as indicated. Following incubation, samples were supplemented with SDS-PAGE loading dye, fractionated by SDS-PAGE, and immunoblotted with MetAP-2 antiserum. (B):Same as (A), except MetAP-2 (9 μM) plus Protac-1 (10 μM) were supplemented with either IκBα phosphopeptide (50μM) or ovalicin (50μM) as indicated. Protac binding to MetAP-2 was inhibited by addition of ovalicin, but not phosphopeptide (B).

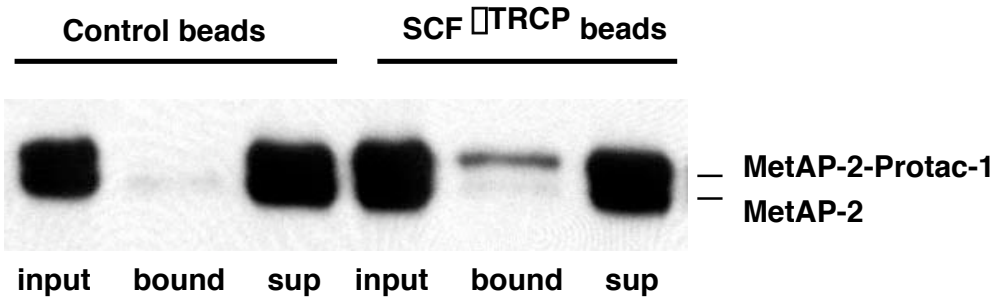
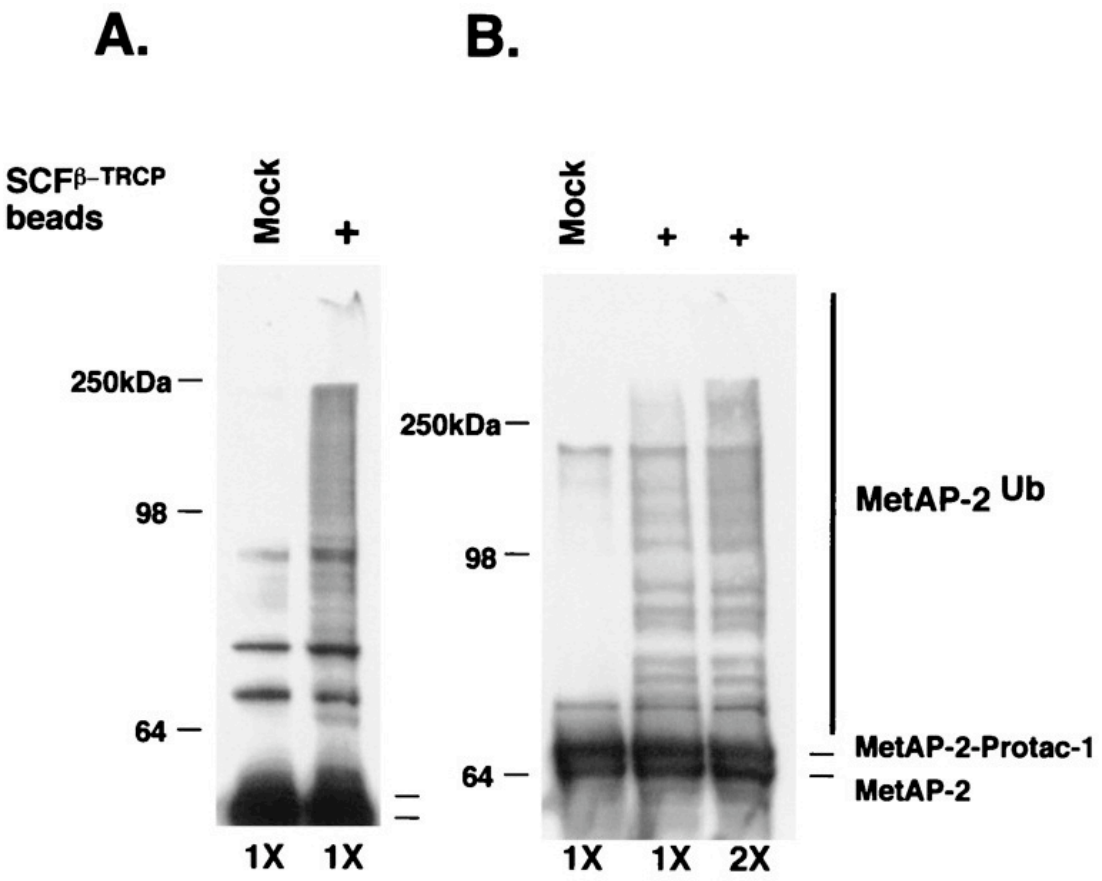
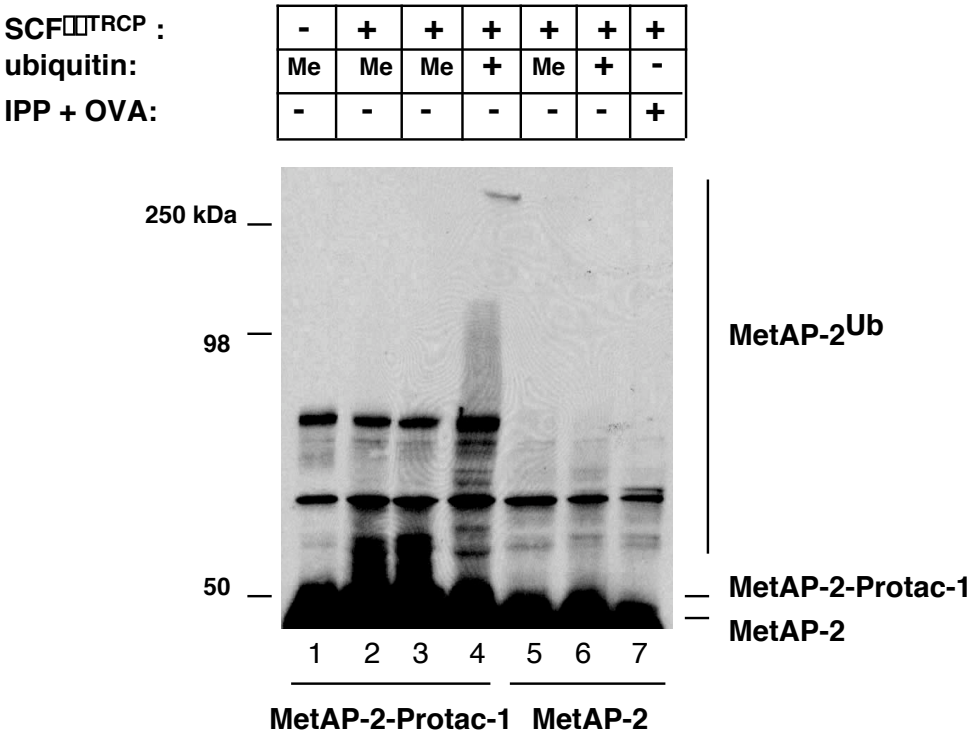


Figure II-3. Protac-1 recruits MetAP-2 to SCF$^{\beta\text{-TRCP}}$.

Extracts from 293T cells transiently transfected with either control vector or plasmids expressing FLAG epitope-tagged Cul1 and β-TRCP were subject to affinity purification on anti-FLAG resin to yield either control beads or SCF$^{\beta\text{-TRCP}}$ beads. The matrices were then mixed with the preformed MetAP-2–Protac-1 complex (input), incubated, and separated into bound (pellet) and unbound (sup) fractions. Proteins were fractionated on an SDS-10% polyacrylamide gel, and immunoblotted with anti-MetAP-2 antiserum. MetAP-2 and MetAP-2–Protac-1 refer to free MetAP-2 and MetAP-2 complexed with Protac-1, respectively. The 1X and 2X represent the relative amount of E1, E2, and ubiquitin added to the reactions.


A.
B.
SCFβ-TRCP beads
Mock
+
250kDa
98
64
1X
1X
Mock
+
+
250kDa
98
64
MetAP-2 Ub
MetAP-2-Protac-1
MetAP-2
1X
1X
2X

C.


SCFβTRCP :
ubiquitin:
IPP + OVA:
- + + + + + +
Me Me Me + Me + -
- - - - - - +
250 kDa
98
50
MetAP-2Ub
MetAP-2-Protac-1
MetAP-2
1 2 3 4 5 6 7
MetAP-2-Protac-1
MetAP-2

D.

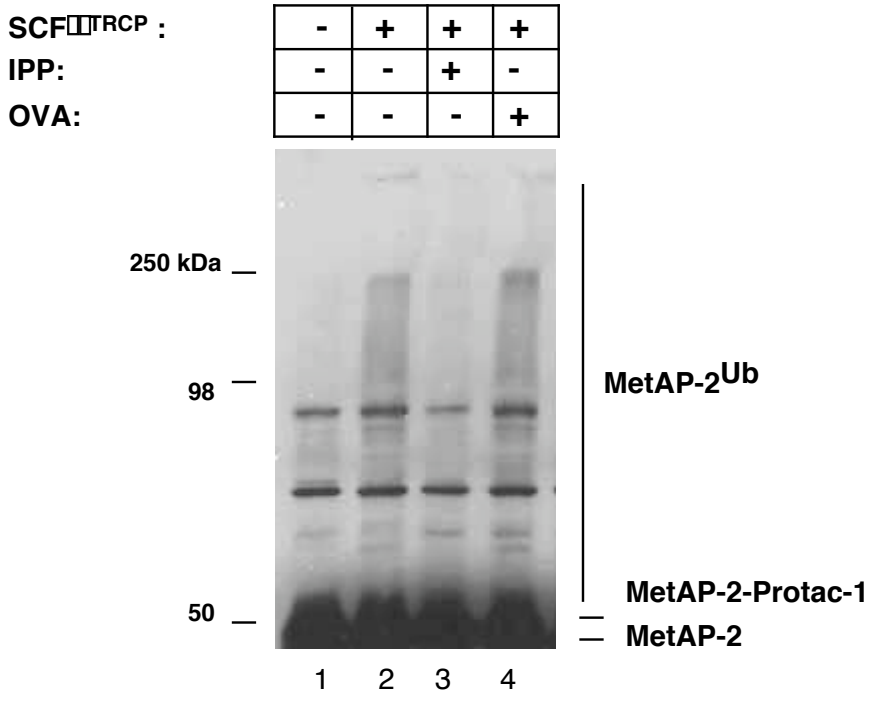

SCFβTRCP :
IPP:
OVA:
- + + +
- - + -
- - - +
250 kDa
98
50
MetAP-2Ub
MetAP-2-Protac-1
MetAP-2
1 2 3 4

Figure II-4. Protac mediates MetAP-2 ubiquitination by SCF.

(A) Ubiquitination of the 46 kDa fragment of MetAP-2. MetAP-2–Protac-1 mixture was added to either control (mock) or $SCF^{\beta\text{-}TRCP}$ beads (+) supplemented with ATP plus purified E1, E2 (Cdc34), and ubiquitin. UbcH5c (500 ng) was also tested as E2 in the reaction, which resulted in the same degree of ubiquitination as observed with Cdc34 (data not shown). Reactions were incubated for one hour at 30°C, and were evaluated by SDS-PAGE followed by western blotting with anti-MetAP-2 antiserum. (B): Ubiquitination of full length (67 kDa) MetAP-2. Same as (A), except that the 67 kDa preparation of MetAP-2 was used, and E1, E2, plus ubiquitin were either added at normal (1X) or two-fold higher (2X) levels, as indicated. (C): Ubiquitination of MetAP-2 by $SCF^{\beta\text{-}TRCP}$ depends upon Protac-1. Same as (A), except that methyl ubiquitin (Me) was substituted for ubiquitin, as indicated, and the reactions depicted in lanes 5-7 lacked Protac-1. In lane 7, unlinked IβBα phosphopeptide (IPP) and ovalicin (OVA) were each added at 100 μM in place of Protac-1. (D): Protac-1 dependent ubiquitination of MetAP-2 is competitively inhibited by IβBα phosphopeptide. Same as (A), except that reactions in lanes 3 and 4 were supplemented with 100 μM each IβBα phosphopeptide (IPP) and ovalicin (OVA), respectively.

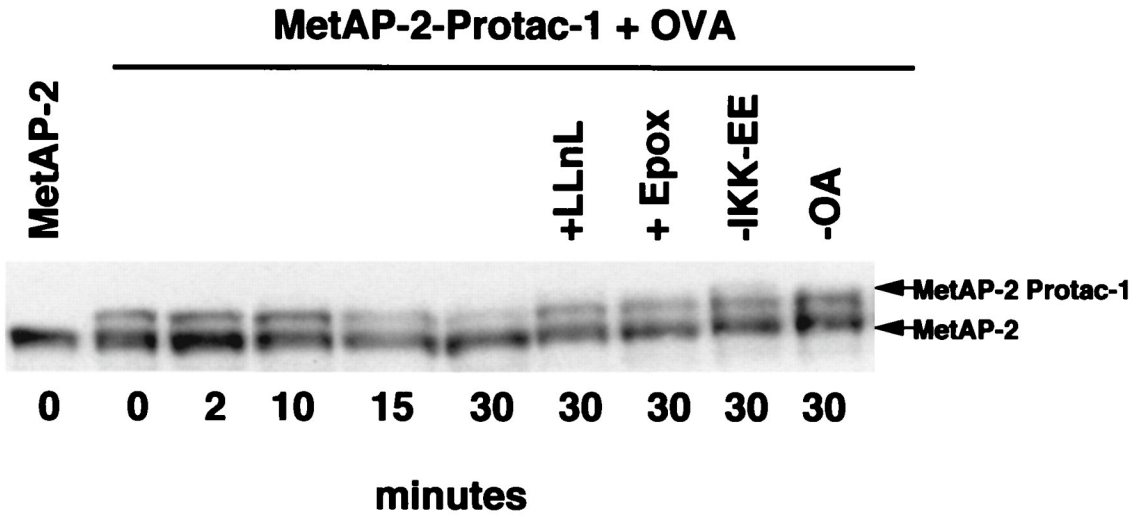


Figure II-5. MetAP-2-Protac is degraded in *Xenopus* extracts.

The MetAP-2–Protac-1 mixture or MetAP-2 alone was added to *Xenopus* egg extract fortified with ovalicin (OVA; 100 □M), IKK-EE (0.4 μg) and okadaic acid (10 □M). Where indicated, reactions were either deprived of IKK-EE or okadaic acid (OA), or were further supplemented with 50 □M LLnL or 10 □M epoxomicin (Epox). Reactions were incubated for the indicated timepoints at room temperature, terminated by adding SDS-PAGE loading dye, and evaluated by SDS-PAGE followed by western blotting with anti-MetAP-2 antiserum.

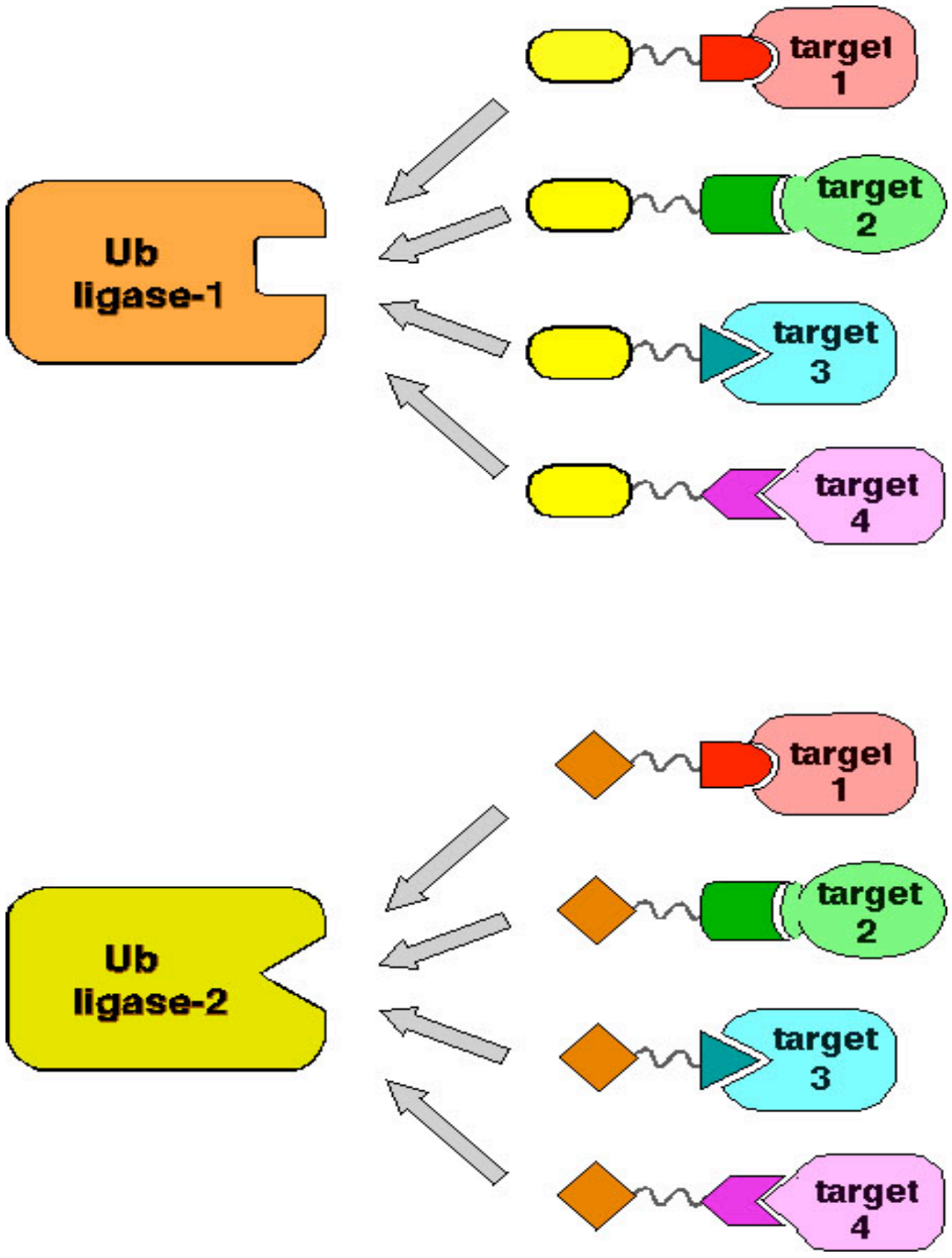


Figure II-6. General application of Protacs. A schematic of how different disease-promoting proteins might be recruited to different ubiquitin ligases for ubiquitination and degradation by unique Protacs.

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Chapter III

Protacs Target MetAP-2 to the Cbl Ligase for Ubiquitination

The results presented in this chapter were generated in collaboration with Craig Crews and Ning Zheng. This work has been previously published (Sakamoto K.M., Mol. Gen. Metab., 2002; see Appendix).

Introduction

The Cbl family of proteins comprises of five known members: three mammalian proteins, c-Cbl, Cbl-b, and Cbl 3, and two from invertebrates Sli-*1 (C. elegans*) and D-Cbl (*D. melanogaster*). There are also two oncogenic forms of Cbl, v-Cbl and 70Z-Cbl. Sli-1 negatively regulates signaling from a receptor tyrosine kinase (RTK). All Cbl proteins have a unique domain that recognizes phosphorylated tyrosine residues that are present on activated tyrosine kinases. Cbl proteins also recruit ubiquitin-conjugating enzymes (E2s or UBCs) to activated RTKs and direct multi-ubiquitinated proteins for degradation (1). Cbl proteins therefore function by specifically targeting activated RTKs and mediating their downregulation, thereby providing a means by which signaling processes can be negatively regulated. Interestingly, Cbl proteins are involved in positive signaling events through its function as a multidomain adaptor protein that is required for functions associated with bone resorption, glucose uptake in response to insulin, and cell spreading in response to integrin engagement (1).

All of the Cbl proteins share a high degree of sequence similarity in the NH2-terminal half, which includes a phosphotyrosine-binding (PTB) domain, a short linker

region, and the RING domain. The PTB domain is composed of a four-helix bundle domain that contains two calcium-binding EF hand motifs, and a domain that adopts the fold of classical Src homology 2 (SH2) domains but has very little sequence similarity to other SH2 domains. The four-helix, EF hand, and SH2 domains all contribute to the PTB function. The COOH-terminal halves of c-Cbl and Cbl-2 contain proline-rich regions, which serve as docking sites for Src homology 3 (SH3) domain-containing proteins, such as growth receptor binding protein 2 (Grb2), Src, and phosphatidylinositol 3-kinase (PI3K), and several tyrosines, which, when phosphorylated by activated tyrosine kinases, bind to the SH2 motifs of various signaling molecules. Finally c-Cbl forms homodimers through the leucine zipper at the very COOH terminal of the protein (1).

Although c-Cbl and Cbl-b-deficient mice are generally healthy and do not show developmental abnormalities, they have a marked defect in thymocyte and peripheral T-cell activation, respectively. Thymocytes in c-Cbl-/- mice show a marked activation of ZAP-70 in response to T cell receptor activation compared to wild type thymocytes. The Cbl-b deficient mice are highly susceptible to autoimmune disease. Thus a lowered threshold for TCR signaling is a common theme in both mutant mice (1).

C-Cbl was identified through the isolation of the oncogenic v-Cbl protein consisting only of the TKB domain. V-Cbl appears to function as a dominant-negative protein by competing with wild-type Cbl proteins for binding sites on activated tyrosine kinases, thus preventing Cbl from negatively regulating these target kinases. This model is supported by the inability of v-Cbl to transform when its TKB domain is mutated (G306E) and can no longer bind activated tyrosine kinases. A mutant form of c-Cbl was isolated from the 70Z/3 mouse pre-B cell lymphoma cells. This mutant protein, called 70Z-Cbl, has

a 17-amino-acid deletion between 366 and 382 that removes most residues in the linker domain that encompass the a-helix, plus the first cysteine of the RING finger. Expression of a Cbl protein with this 17-amino-acid deletion, or deletion of either of two tyrosine residues within the a-helix of the linker induces more rapid and acute transformation of NIH 3T3 cells than does v-Cbl (1,2).

Recently, the structure of the complex between c-Cbl and the E2 UBCH7 has been elucidated (3, 4). Furthermore, the ZAP-70 phosphopeptide and recognition site of Cbl were determined and found to be evolutionarily conserved (5, 6). Based on the crystal structure of the c-Cbl/UbcH7/phospho-Zap-70 peptide complex, the linker interacts with both UbcH7 and the TKB domain of c-Cbl and thus its mutations may disrupt both E2-dependent activity and PTK-binding ability of c-Cbl.

To test the concept of Protac in a more generalizable manner, we sought to use c-Cbl as another ubiquitin ligase in place of $SCF^{\beta\text{-}TRCP}$. A Protac was generated consisting of the minimal ZAP-70 phosphopeptide and the MetAP-2 binding protein, ovalicin. We first tested whether the Zap-70-ovalicin Protac could inhibit ubiquitination of a known substrate of c-Cbl. Next, we determined whether MetAP-2 binds Zap-70-ovalicin Protac in a specific, concentration dependent manner. Finally, we performed experiments that would demonstrate that c-Cbl ubiquitinates MetAP-2 in the presence of Zap-70-ovalicin Protac.

Results

Zap-70-ovalicin inhibits Cbl ligase activity

A Protac containing a Zap-70 phosphopeptide at one end and ovalicin moiety at the other end was generated to test whether MetAP-2 could be recruited to Cbl for

ubiquitination. We first tested whether the Zap-70-ovalicin could inhibit ubiquitination of a known substrate of Cbl, "X". Increasing concentrations of Protac and the Zap-70 phosphopeptide alone inhibited the ubiquitination of substrate "X" (Fig. 1). However, the concentration of Protac (100 μM) required to inhibit ubiquitination of "X" was higher than the concentration (10 μM) required for IκBα phosphopeptide or Protac to inhibit IκBα ubiquitination. Therefore, the Zap-70 phosphopeptide and Protac specifically inhibits the ubiquitination of a known substrate of Cbl in a concentration-dependent manner.

MetAP-2 specifically binds Zap-70-ovalicin Protac *in vitro*

To demonstrate that purified MetAP-2 bound Zap-70 Protac, we incubated MetAP-2 (18μM) with increasing concentrations of Protac (Fig. 2). A Western blot analysis was performed with anti-MetAP-2 anti-serum. At 200 μM Protac and above, we observed two bands; the lower band represents unbound MetAP-2, and the upper band represents MetAP-2 bound to Protac. Addition of Protac at higher concentrations yielded a third band that is possibly a result of dimerization of MetAP-2. Combining MetAP-2 with either free Zap-70 phosphopeptide or free ovalicin did not yield the doublet observed with the Protac. We also tested the specificity of MetAP-2 interaction with Protac *in vitro*. Free ovalicin, but not free Zap-70 phosphopeptide, inhibited the formation of the MetAP-2 Protac complex. Therefore, our results demonstrate that MetAP-2 was specifically conjugated to the ovalicin moiety of Protac in a concentration-dependent manner.

Zap-70-ovalicin Protac mediates the ubiquitination of MetAP-2

To test whether Protac mediates MetAP-2 ubiquitination by Cbl, we performed experiments with MetAP-2, Cbl, ubch4 (E2), and several E1s, including GST-E1, yeast E1 and Rabbit UBA1 (Boston Biochem, Inc.). MetAP-2 was ubiquitinated by Cbl in a Protac-

dependent manner (Fig. 3). In the absence of Protac, we did not observe modification of MetAP-2. These results demonstrated that the Zap-70-ovalicin recruited MetAP-2 to Cbl, resulting in ubiquitination. Therefore, Protacs can be generalized to target proteins to ubiquitin ligases other than $SCF^{\beta\text{-}TRCP}$.

Discussion

The goal of these experiments was to further test our hypothesis that a chimeric molecule could recruit and target a stable protein not previously known to be a substrate of a ubiquitin ligase could be deliberately ubiquitinated. We showed in Chapter II that MetAP-2 could be ubiquitinated by the $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase. In this chapter, we hoped to determine whether the ubiquitin ligase Cbl could also ubiquitinate MetAP-2 in the presence of the chimeric molecule or Protac (Protac). Our previous results showed that $SCF^{\beta\text{-}TRCP}$ is a promiscuous enzyme that is able to ubiquitinate MetAP-2 (7). In this Chapter, we observed that Cbl could also ubiquitinate a "foreign" protein that is not known to be a substrate. However, we observed that the Zap-70-phosphopeptide and the Protac had decreased affinity for Cbl compared to the previous Protac-1 for $SCF^{\beta\text{-}TRCP}$ described in chapter II. Furthermore, the affinity of the Zap-70-ovalicin Protac for MetAP-2 was lower than the IβBα-ovalicin Protac-1. These results suggest that certain Protacs may function better than others, depending on the ligase and the target, requiring further optimization. We also observed that Cbl could ubiquitinate MetAP-2 in the presence of different E1, including GST-E1, Rabbit UBA1, and Yeast E1. Future studies will focus on employing Protacs to target different proteins as substrates of ubiquitin ligases. Chapter IV addresses whether Protacs recruit proteins that associate through noncovalent interactions *in vitro* and

in vivo. Ultimately, our goal is to develop a repertoire of Protacs that will bind to desired disease-promoting protein for ubiquitination and degradation in tumor cells.

Experimental Procedures

Synthesis of Zap70-ovalicin Protac

Ovalicin (1.4 mmol) was dissolved in 10 mL of methanol at 0 ^{o}C and $NaBH_4$ (3.0 mmol) was added slowly. After 30 min stirring, methanol was removed under reduced pressure and the resulting crude product was purified by flash column chromatography to yield ovalicinol (1.15 mmol, 82 %). To the ovalicinol, Fmoc-Gly was coupled to give Fmoc-Gly-ovalicinol. Specifically, dimethylformamide (DMF) (28 μL) was added to dichloromethane solution (30 mL) containing Fmoc-Gly-OH (3.56 mmol) and oxalyl chloride (7.12 mmol) at 0 ^{o}C. After 3 hour stirring at room temperature, dichloromethane was removed under nitrogen atmosphere. The resulting solid residue was redissolved in dichloromethane (10 mL) and was combined with ovalicinol (0.6 mmol) and dimethylamino pyridine (DMAP) (4.7 mmol) in dichloromethane (30 mL) at 0 ^{o}C. The reaction mixture was stirred for 2 hours at room temperature. After dichloromethane was removed under reduced pressure, the resulting residue was flash chromatographed to provide Fmoc-Gly-ovalicinol (0.39 mmol, 65 %). Next, Fmoc-Gly-ovalicinol (0.09 mmol) was treated with 20 % piperidine in DMF (2 mL) at room temperature for 10 minutes and DMF was removed under high vacuum. The resulting solid was redissolved in 2 mL of dimethylsulfoxide (DMSO) and disuccinimidyl suberate (DSS) (0.9 mmol) was added at room temperature. After overnight stirring, DMSO was removed under high vacuum and the resulting crude product was flash chromatographed to give monosuccinimidyl suberate-

Gly-ovalicinol (0.06 mmol, 68 %). Monosuccinimidyl suberate-Gly-ovalicinol (12 μmol) in DMSO (0.6 mL) was added to DMSO solution (1 mL) containing Zap70 peptide (3.67 μmol) and DMAP (11 μmol). After 20 min stirring at room temperature, the coupling reaction was completed, which was confirmed by Kaiser test. DMSO was removed under high vacuum and the resulting crude product was repeatedly washed with dichloromethane and methanol to remove excess monosuccinimidyl suberate-Gly-ovalicinol to give the final product (Zap70 peptide-suberate-Gly-ovalicinol) (5.8 mg, 2.59 μmol, 70 %). The final product was characterized by electrospray (ES) mass spectrometry. ES-MS $(M+H)^+$ for ovalicinol-Gly-suberate-Zap70 peptide:DMAP, 2,231.56. All other intermediates were characterized by 500 MHz ^{1}H NMR spectroscopy.

MetAP-2–Protac-1 coupling assay

MetAP-2 (9 □M) was incubated with increasing concentrations of Protac (dissolved in water) at room temperature for 45 minutes. Reactions were supplemented with SDS loading dye, fractionated on a 10% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane and immunoblotted with rabbit polyclonal anti-MetAP-2 antiserum (Zymed, Inc.). ECL was performed using Amersham detection reagents.

Ubiquitination assays

For experiments with Cbl ubiquitination of "X" in the presence of Zap70 phosphopeptide as competitor, "X" was phosphorylated in the presence of 32P□ATP at 37^{o}C for 30 minutes and added to reaction with E1, E2, and Cbl. For ubiquitination reactions, 4 □l of 18 μM MetAP-2 preincubated with 4 □l of 100 μM Protac, 0.5 □l of 0.1 μg/µl purified rabbit, yeast or GST-E1 1 □l, 0.5 μg/μl ubch4 E2, and 1□l 25 mM ATP were

added to 10 μl (packed volume) of purified Cbl (kindly provided by Ning Zheng). For competition experiments, the phosphopeptide (100 μM final) or ovalicin (100 μM final) was added simultaneously with the Protac-1. Reactions were incubated for one hour at 30°C. SDS-PAGE loading dye was added to terminate reactions, which were evaluated by western blot analysis.

Acknowledgements

I would like to thank Rati Verma for helpful suggestions, Craig Crews for providing the Protacs, and Ning Zheng for providing purified Cbl, ubch4, and GST-E1 for ubiquitination assays. This work was supported by CaPCURE and the UC BioSTAR Project (biostar 01-10232). I would also like to thank Frank Mercurio and Celgene, Signal Research Division for supporting this work and providing reagents.

Figures

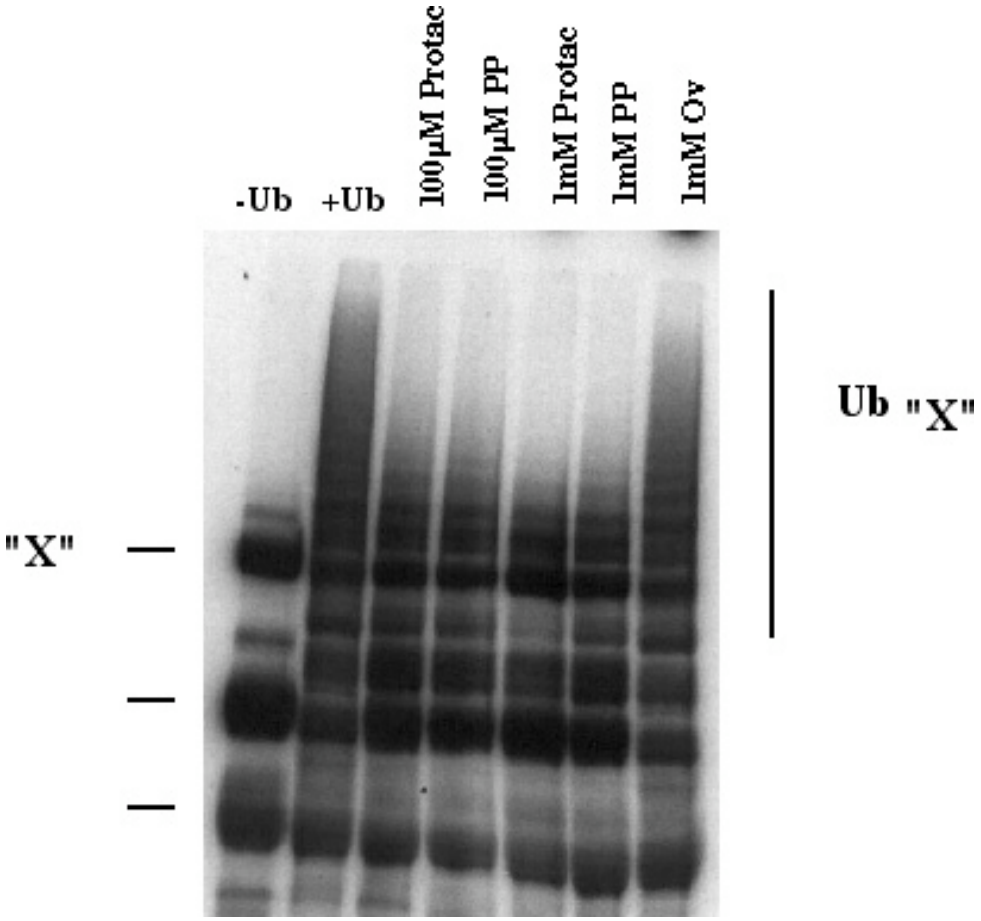

Figure III-1. The Zap-70-ovalicin Protac (Protac) and Zap-70 phosphopeptide inhibit ubiquitination of Cbl substrate "X." "X" was phosphorylated with kinase in the presence of 32P ATP, then incubated with Cbl, ubch4, ubiquitin, and Protac or phosphopeptide. These results demonstrate that the Zap-70 Protac inhibits Cbl ubiquitination of "X."

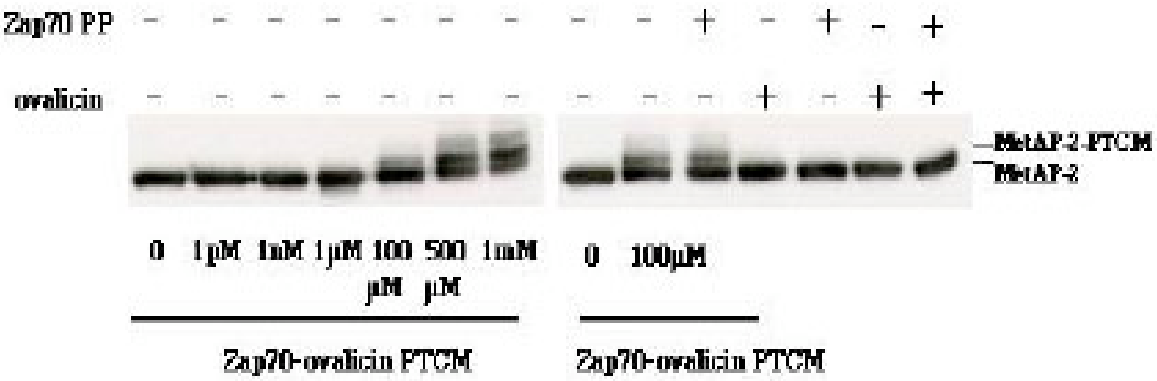

Figure III-2. MetAP-2 binds Zap-70-ovalicin Protac (PTCM=Proteolysis Targeting Chimeric Molecule) specifically in a concentration-dependent manner. MetAP-2 (9 □M) was incubated with increasing concentrations of Protac at room temperature for 45 min. The last two lanes on the right depict MetAP-2 that was incubated with either free Zap-70 phosphopeptide or free ovalicin. Protac binding to MetAP-2 was inhibited by the addition of ovalicin, but not by the addition of the Zap-70 phosphopeptide.

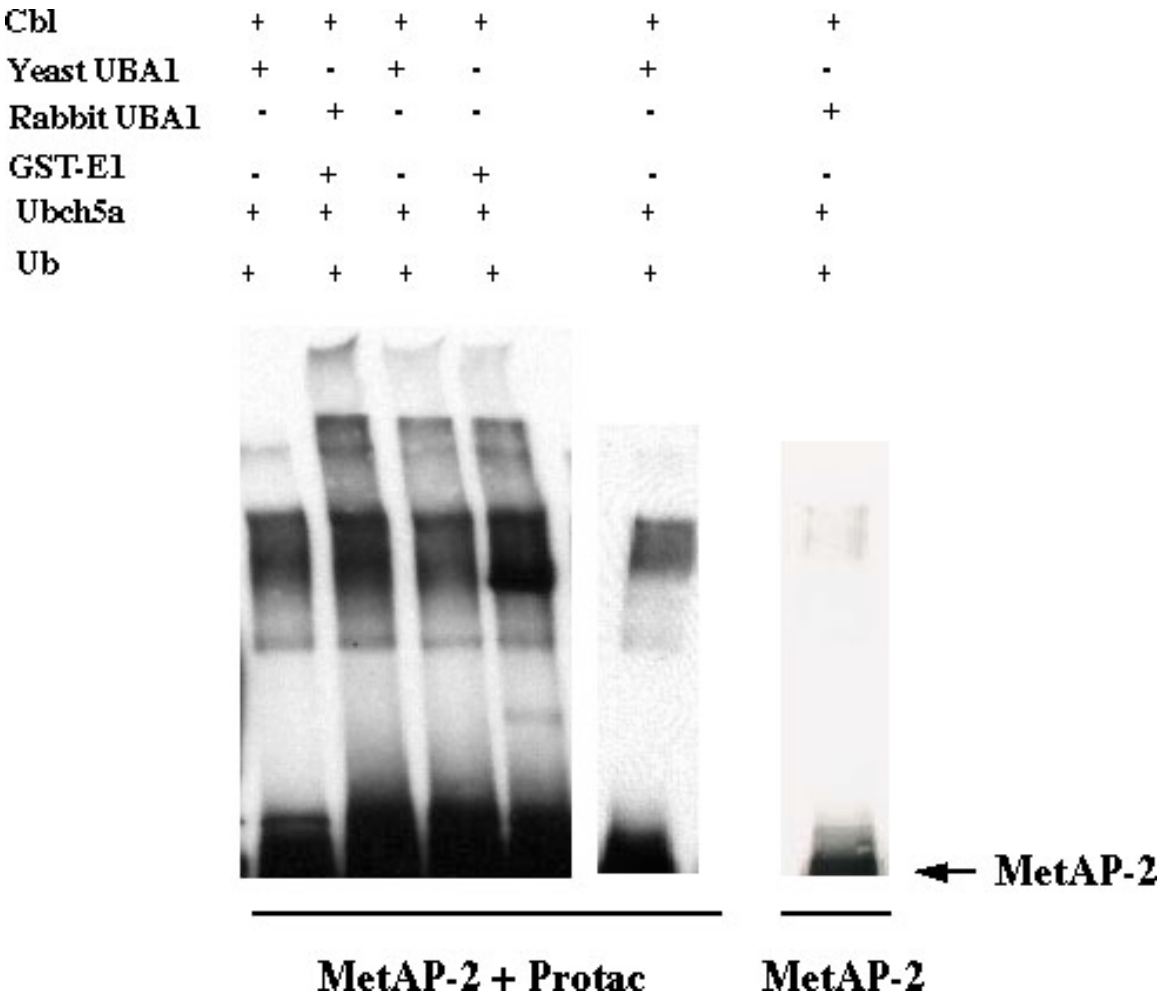

Figure III-3. Ubiquitination of MetAP-2 by Cbl. MetAP-2 bound to Zap70 phosphopeptide-ovalicin was added to purified Cbl, with E1, and E2 (Ubch4), and ubiquitin (ub). The reaction was incubated for 30 minutes at room temperature. Western blot analysis was performed with anti-MetAP-2 antisera. These results suggest that Protac technology can be applied to other ubiquitin ligases.

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Chapter IV

Development of Protacs to Target Cancer-Promoting Proteins for Ubiquitination and Degradation

The data presented in this chapter were generated in collaboration with Kyung B. Kim, Rati Verma, Andy Ransick, Bernd Stein, and Craig M. Crews. This work was previously published (Sakamoto et al., 2003; Appendix).

Introduction

One of the major pathways to regulate protein turnover is ubiquitin-dependent proteolysis. Post-translational modification of proteins with ubiquitin occurs through the activities of ubiquitin activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), which act sequentially to catalyze the attachment of ubiquitin to lysine residues in an energy-dependent manner (1,2). Among the hundreds of E3s encoded within the human genome, the SCF ubiquitin ligases comprise a heterotetrameric group of proteins consisting of Skp-1, Cul1, a RING-H2 protein Hrt1 (also known as Roc1 or Rbx1) and an F-box protein (1,3). The mammalian F-box protein b-TRCP of $SCF^{\beta\text{-}TRCP}$ binds IβBα, the negative regulator of NFκB, and promotes its ubiquitination and degradation (4). A 10-amino-acid phosphopeptide segment of IβBα is both necessary and sufficient to mediate its binding to $SCF^{\beta\text{-}TRCP}$ and subsequent ubiquitination and degradation (4).

There is a pressing unmet need to develop effective drugs to treat cancer and other diseases that afflict humans. The recent completion of the human genome sequence coupled with basic studies in molecular and cellular biology have revealed hundreds to thousands of proteins that could conceivably serve as targets for rational drug therapy. Unfortunately, many of these protein targets are not considered to be readily "drugable", in that they are not enzymes and it is not obvious how to inhibit their function with small molecule drugs. Thus, it would be valuable to have a generic method that would enable specific and efficacious inhibition of any desired protein target, regardless of its biochemical function. Short interfering RNA (siRNA) represents one such method (5, 6), but it remains unclear whether siRNA will work as therapeutic agents in humans. We sought to develop a different approach, taking advantage of the 10-amino acid phosphopeptide sequence of I□B□ described above to target proteins for ubiquitination and degradation (4).

As proof of concept, we previously synthesized a chimeric molecule or Protac (Proteolysis Targeting Chimeric molecule) consisting of the I□B□ phosphopeptide linked to ovalicin, which covalently binds methionine aminopeptidase-2 (Met-AP-2). We showed that this Protac (Protac-1) recruits MetAP-2 to the $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase resulting in both ubiquitination and degradation of Met-AP2 (7). MetAP-2 is not known to be an endogenous substrate of $SCF^{\beta\text{-}TRCP}$ (8), and was not ubiquitinated by $SCF^{\beta\text{-}TRCP}$ in the absence of Protac-1. Although this experiment demonstrated that Protacs could work as envisioned, it left open a number of critical questions. For example, can Protacs be used more generically to target other substrates, including proteins of potential therapeutic interest? Can a Protac recruit a target to $SCF^{\beta\text{-}TRCP}$ through a noncovalent interaction? Can a Protac work within the context of a cell?

Both estrogen receptor alpha (ER□) and androgen receptor (AR) have been demonstrated to promote the growth of breast and prostate cancer cells (9, 10). In fact, there are several treatment modalities such as Tamoxifen and Faslodex, which control breast tumor cell growth through inhibition of ER activity. In early prostate cancer, tumor cells are often androgen-responsive. Patients with prostate cancer receive hormonal therapy to control tumor growth. Recent evidence suggests that even in androgen-independent prostate cancer, the AR may promote tumor growth (10). Similarly, many tamoxifen-resistant tumors still express ER (11). Thus, new drugs that downregulate AR and ER by novel mechanisms may be of potential benefit in treating breast and prostate cancers.

To address the key questions about Protacs raised by our first study, we set out to develop Protacs comprising the I□B□ phosphopeptide linked to either estradiol or dihydroxytestosterone (DHT) to recruit ER or AR to SCF□-TRCP to accelerate their ubiquitination and degradation. Recently, both the ER and AR have been shown to be regulated by proteasome-dependent proteolysis (12-14). We reasoned that Protacs might mimic the action of the human papillomavirus E6 protein, which accelerates the turnover of the already unstable p53 to the point where p53 can no longer accumulate, resulting in loss of its function (15).

In this paper, we report the feasibility of using Protacs to target degradation of proteins known to promote tumor growth. We show that Protacs can recruit the ER for ubiquitination and degradation in a cell-free system. Furthermore, our results demonstrate that in cells, Protacs can promote the degradation of AR in a proteasome-dependent manner. Thus, Protacs may be a useful therapeutic approach to destroy proteins that promote tumor growth in patients with cancer.

Results

Protacs consisting of the minimal 10 amino acid peptide (phosphorylated on the underlined S residues), DRHDSGLDSM covalently linked to either estradiol (E2; Protac-2) or dihydroxytestosterone (DHT; Protac-3) were synthesized (Fig. 1). We first performed *in vitro* ubiquitination assays with both Protacs, but focused our efforts on Protac-2 due to problems encountered with expression of recombinant AR. To determine whether Protac-2 promotes the ubiquitination of ER by SCF□-TRCP in a concentration-dependent manner, we performed ubiquitination assays with increasing concentrations of Protac (Fig. 2A). ER

was ubiquitinated starting at a concentration of 0.1 to 1mM Protac-2, with maximal efficiency observed at 5□M to 10□M. At 500 □M, we no longer observed ubiquitination of ER by SCF□-TRCP, which may be due to a 'squelching' phenomenon wherein the presence of excess Protac-2 inhibits competitively the formation of heteromeric ER–Protac-2–SCF complexes. Since 10 □M Protac-2 promoted efficient ubiquitination of ER, we continued to use this concentration for the remainder of our studies (except as noted below). It should be noted that we consistently observed Cul1-dependent ubiquitination of ER in the absence of Protac-2 (e.g., Fig. 2A and 2B, lane 1). This may be due to the presence of an ER-specific SCF ubiquitin ligase in the Cul1 precipitates. Regardless, these Protac-independent conjugates were of low molecular weight and clearly distinguishable from the high molecular weight, methyl ubiquitin-sensitive conjugates induced by Protac-2 (e.g., compare lanes 1, 3, and 4 of Fig. 2B).

To address the mechanism of action of Protac-2, we tested whether the IkBa phosphopeptide and estradiol individually can compete out Protac-2, and whether these ligands when added together as free compounds can mimic the action of Protac-2. A 10-fold excess of either I□B□ phosphopeptide (Fig. 2D) or estradiol (Fig. 2E) in cells completely blocked the ubiquitination-promoting activity of 1□M Protac-2. Moreover, when added together as separate compounds, estradiol and I□B□ phosphopeptide failed to reproduce the effect of Protac-2 (Fig. 2C).

These results are consistent with our hypothesis that Protac-2 acts as a bridging molecule in that the estradiol moiety associates with the ER while the other moiety, the I□B□ phosphopeptide, recruits the ER to the SCF□-TRCP.

We next tested the specificity of Protac-mediated ubiquitination. Ubiquitination assays with ER were performed in the presence of either Protac-2, Protac-3 or a Protac (Protac-4) that consisted of the Zap70 phosphopeptide, which is recognized by the Cbl ubiquitin ligase (16) and ovalicin, which binds Met-AP-2 (8). As shown in Fig. 2F, ER was not ubiquitinated by $SCF^{\beta\text{-}TRCP}$ in the presence of either Protac-3 or Protac-4.

Not all ubiquitin-ubiquitin linkages are able to sustain targeting to the proteasome (17), and possibly as a consequence, substrates ubiquitinated under the relatively artificial conditions encountered in reconstituted systems can be poor substrates for the proteasome (18). Thus, we sought to determine whether ER-ubiquitin conjugates induced by Protac-2 were recognized by the 26S proteasome. To answer this question, purified yeast 26S proteasome (19) was added to ubiquitinated ER formed in the presence of $SCF^{\beta\text{-}TRCP}$ and Protac-2. Complete disappearance of high MW ubiquitin conjugates was observed within 10 minutes (Fig. 3A) and was partially blocked by the metal chelator 1,10 phenanthroline (which inhibits the essential Rpn11 isopeptidase activity of the proteasome), but not by the inactive derivative 1,7 phenanthroline (20) (Fig. 3B).

Our results with the I□B□ phosphopeptide-estradiol Protac demonstrated that a medically relevant target protein can be recruited to a ubiquitin ligase through noncovalent interactions and be ubiquitinated and degraded *in vitro*. We next wished to test whether a Protac could promote the degradation of proteins in cells. For these experiments we used Protac-3, because we encountered technical difficulties in working with cells that transiently expressed an ER-based reporter protein and because a 293 cell line that stably expresses AR-GFP ($293^{AR\text{-}GFP}$) was readily available to us. We employed microinjection because the phosphate groups on the IkBa phosphopeptide preclude its efficient uptake into cells. $293^{AR\text{-}GFP}$ cells were injected with Protac-3 (10 □M stock; 1□M final) and monitored for presence or absence of GFP by fluorescence microscopy. A time course was performed, and maximal GFP-AR degradation was observed one hour after injection of Protac (data not shown; Fig. 4A). We observed that the majority of cells injected with Protac expressed decreased levels of GFP (Fig. 4B). This decrease was not due to GFP-AR leakage since cells coinjected with rhodamine were not affected after one hour (indicated by the pink stained cells shown in Fig. 4). To quantify the degree of GFP-AR degradation, we counted over 200 cells and determined the relative decrease in GFP-AR signal one hour following injection (Fig. 4B). Greater than 70% of cells demonstrated minimal, partial, or complete disappearance of GFP-AR. In all experiments, only cells that continued to be rhodamine positive after one hour were counted. Each experiment was performed on at least two separate days with 30-50 cells injected per experiment. Injection of rhodamine or 200 mM KCl buffer alone did not result in disappearance of GFP from $293^{AR\text{-}GFP}$ cells (data not shown).

We further verified that the linkage of phosphopeptide and DHT was required for GFP-AR degradation. Coinjection of free I□B□ phosphopeptide and testosterone (10□M each) into 293 cells did not result in decreased GFP signal (Fig. 4C), indicating that intact Protac is necessary to promote degradation of GFP-AR. To determine whether GFP-AR degradation was dependent on I□B□ phosphopeptide and testosterone binding to their respective targets, we coinjected Protac-3 (10mM) with a 10-fold molar excess (100mM) of free phosphopeptide (Fig. 4D) or testosterone (Fig 4E) into $293^{AR\text{-}GFP}$ cells. In both cases, degradation of GFP-AR was inhibited. All experiments were performed on 3 separate days with 20 to 30 cells injected per experiment. The results shown are representative of the phenotype in greater than 70% of cells counted. Taken together, these data support the hypothesis that Protac-3 induced AR-GFP degradation by targeting AR-GFP to $SCF^{\beta\text{-}TRCP}$.

To determine whether the disappearance of GFP-AR was proteasome dependent, $293^{AR\text{-}GFP}$ cells were treated with the proteasome inhibitor epoxomicin for 4 hours prior to injection with Protac-3 (10 mM)(Fig. 4F). In cells treated with epoxomicin, GFP-AR was not degraded, suggesting that the Protac mediates degradation through a proteasome-dependent pathway. Cells were also coinjected with Protac (10□M) and epoxomicin (10□M) in the absence of pretreatment resulting in inhibition of GFP-AR degradation (data not shown). The result shown is representative of experiments performed on three different days with at least 30 cells injected per day.

As demonstrated previously (Fig. 2F), the I□B□ phosphopeptide-estradiol Protac-2, but not Protac-3, specifically induces ubiquitination of ER *in vitro*. The specificity is dependent on the ability of Protac-2 to be recognized by the ubiquitin ligase as well as its ability to bind to ER. The same specificity of Protac action appears to hold true in cells, because Protac-2, unlike Protac-3, does not induce degradation of GFP-AR (Fig. 4G)

Discussion

The ubiquitin-proteasome pathway rapidly, efficiently, and selectively ubiquitinates and degrades targeted polypeptides. Many signaling processes critical to the biology of normal and diseased cells are regulated by ubiquitin-dependent proteolysis, including exit from M phase of the cell cycle and initiation of innate immune response, which are respectively controlled by degradation of cyclin B and the NF-□B regulator I□B□ (21, 22). To harness the power of the ubiquitin-proteasome pathway for therapeutic purposes, we are developing 'Protacs' to recruit proteins to ubiquitin ligases to promote their ubiquitination and degradation. An important aspect of the Protacs approach is that it in theory can be applied to any protein in the cytoplasm or nucleus of a diseased cell, and thus may enable the development of therapeutics against a large fraction of proteins in the proteome. The linchpin of our approach is a heterobifunctional small molecule (i.e., Protac) that serves as a bridge to link a target protein to a ubiquitin ligase. Previously, we demonstrated that a Protac comprising a phosphopeptide that binds $SCF^{\beta\text{-}TRCP}$ and a small molecule (ovalicin) that binds MetAP-2 activates the ubiquitination of MetAP-2 by $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase *in vitro*, and consequently targets MetAP-2 for degradation by the proteasome in frog extract (7).

Our goals in the current work were to show that Protacs can increase the turnover of a given target protein in cells, and to extend the Protacs approach to proteins that play a causal role in human diseases. We chose the estrogen and androgen receptors for our current studies due to their well-characterized association with estrogen and androgen, respectively. Furthermore, both receptors have been associated with the development and progression of cancer.

The results reported here indicate that Protacs operate by a bridging mechanism to enable efficient and specific downregulation of ER *in vitro* and AR in cells. From our *in vitro* data, it is apparent that Protacs can be developed against different targets (MetAP-2, ER, and AR), and that Protacs promote ubiquitination of these targets in a manner that is both target-selective and dose-dependent.

From microinjection experiments, it is clear that Protacs can activate AR turnover in the context of the cellular degradation machinery. This degradation was also found to be specific and dependent on both components of the Protac molecule. Moreover the proteasome inhibitor epoxomicin blocked the ability of Protacs to promote AR turnover, suggesting that the degradation is proteasome-specific and not due to alternative pathways, such as those involving lysosomes, or due to other proteases, such as caspases.

To deliver Protacs to cells in the experiments described here, we employed microinjection due to the impermeability of the SCF$^{\beta\text{-TRCP}}$-binding Iβ phosphopeptide moiety. A key remaining challenge for Protac technology is to develop cell permeable molecules that can be used to test for efficacy in cell and animal models of cancer. Ongoing work in our laboratories suggests that Protacs based on the hydroxyproline motif of HIF1-a may be used to target ubiquitination and degradation of proteins in cells through the VHL ubiquitin ligase pathway (manuscript in preparation).

We postulate that many Protac compounds can be generated to treat a variety of diseases. First of all, hundreds of putative ubiquitin ligases that can be exploited as agents of Protac action have been uncovered by the Human Genome Project. Second, it is important to note that Protacs should not be limited to receptors with well-defined ligands such as AR and ER. In theory, any protein that binds a small molecule through high affinity interactions can be a candidate target. Our studies suggest that Protacs technology is not only feasible, but warrants further exploration as an alternative to conventional pharmacologic inhibition of proteins that promote human disease. Current treatment of cancer includes drugs that nonspecifically inhibit the cell cycle, DNA repair, and metabolism. Protacs provide a means of specifically targeting a protein that is known to regulate abnormal growth and survival of cancer cells, in much the same way that Gleevec improves the survival of CML patients by inhibiting the causative agent BCR-ABL (23). The hope is that by developing a generic method that enables us to target the proteins responsible for the malignant phenotype, regardless of their mechanism of action or functional attributes, it will be possible to combat cancer while sparing damage to normal cells.

Experimental Procedures

Synthesis of Protacs

IκBα phosphopeptide-estradiol Protac. To generate GA-1-monosuccinimidyl suberate, the estradiol derivative, GA-1 , (7 mg, 11.5 μmol) was dissolved in 1 ml of anhydrous DMF, and disuccinimidyl suberate (21 mg, 57.0 mmol) was added at room temperature. After overnight stirring, DMF was removed under high vacuum, and the resulting white solid was flash-chromatographed to give GA-1-monosuccinimidyl suberate (6.3 mg, 7.3 μmol, 63.5 %). For synthesis of GA-1-IκBα phosphopeptide, GA-1-monosuccinimidyl suberate (6 mg, 6.9 mmol) in DMSO (1 ml) was added to DMSO solution (0.4 ml) containing IκBα phosphopeptide (1.5 mg, 0.92 mmol) and dimethylaminopyridine (0.5 mg). After 30 min stirring at room temperature, the coupling reaction was completed, which was confirmed by a Kaiser test. DMSO was removed under high vacuum, and the resulting crude product was repeatedly washed with dichloromethane and methanol to remove excess GA-1-monosuccinimidyl suberate to give the final product, GA-1-IκBα phosphopeptide (1.5 mg, 0.63 mmol, 68.5%). The final product was characterized by electrospray (ES) mass spectrometry. ES-MS $(M + H)^+$ for GA-1-IκBα phosphopeptide was 2384.0 Da. All other intermediates were characterized by 500-MHz ^1H NMR spectroscopy.

IκBα-DHT Protac. For DHT-Gly-monosuccinimidyl suberate, dimethylformamide (DMF, 28 μl, 0.33 mmol) was added to dichloromethane solution (20 ml) containing Fmoc-Gly-OH (1.06 g, 3.57 mmol) and oxalyl chloride (0.62 mL, 7.10 mmol) at 0°C. After 3 hr of stirring at room temperature, dichloromethane was removed under nitrogen atmosphere.

The resulting solid residue was redissolved in dichloromethane (8 ml) and was combined with 5a-dihydrotestosterone (0.18 g, 0.62 mmol) and dimethylaminopyridine (0.58 g, 4.75 mmol) in dichloromethane (20 ml) at 0°C. The reaction mixture was stirred overnight at room temperature. After dichloromethane was removed under reduced pressure, the resulting residue was flash-chromatographed to provide DHT-Gly-Fmoc (0.21 g, 0.37 mmol, 60%). Next, DHT-Gly-Fmoc (0.12 g, 0.21 mmol) was treated with tetrabutylammonium fluoride (TBAF, 0.3 mL, 1M in THF) at room temperature for 20 minutes, and the DMF was removed under high vacuum. The resulting residue was flash-chromatographed to provide DHT-Gly-NH_2 (white solid, 49 mg, 0.14 mmol, 67%). Next, disuccinimidyl suberate (0.27g, 0.73 mmol) was added to DMF solution (1 mL) containing DHT-Gly-NH_2 (49 mg, 0.14 mmol) at room temperature. After overnight stirring, DMF was removed under high vacuum, and the resulting crude product was flash-chromatographed to give DHT-Gly-monosuccinimidyl suberate (70 mg, 0.12 mmol, 86%). DHT-Gly-monosuccinimidyl suberate (5.5 mg, 9.16 mmol) in DMSO (0.6 ml) was added to DMSO solution (1 ml) containing I□B□ phosphopeptide (4.5 mg, 2.75 μmol) and dimethylaminopyridine (2.0 mg, 16.37 □mol). After 20 minutes of stirring at room temperature, the coupling reaction was completed, which was confirmed by a Kaiser test. DMF was removed under high vacuum, and the resulting crude product was repeatedly washed with dichloromethane and methanol to remove excess DHT-Gly-monosuccinimidyl suberate to give the final product, DHT-IkBa phosphopeptide (3.5 mg, 1.65 mmol, 60%). The final product was characterized by electrospray (ES) mass spectrometry. ES-MS $(M + H)^+$ for fumagillol-Gly-suberate-HIF-1□ octapeptide was 2,120 Da. All other intermediates were characterized by 500-MHz ^1H NMR spectroscopy.

Tissue culture and transfections

293T cells were cultured in DMEM with 10% (vol/vol) FBS (GIBCO), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2mM). Cells were split 1:5 the day prior to transfection and transiently transfected with 40 μg of plasmid. Cells were 70% confluent in 100-mm dishes on the day of transfection. Cells were transfected with DNA [20 μg of pFLAG-Cul1 (RDB1347) and 20 μg of pFLAG-b-TRCP (RDB1189)] using calcium phosphate precipitation method as described (7). Cells were harvested 30 hours after transfection. Five micrograms of pGL-1, a plasmid containing the cytomegalovirus (CMV) promoter linked to the green fluorescent protein (GFP) cDNA, was cotransfected into cells at the same time to assess transfection efficiency. Cells were greater than 80% GFP positive at the time of harvesting.

Ubiquitination assays with ER

293T cell pellets were lysed with 200 μl of lysis buffer (25 mM Tris-Cl, pH 7.5/150mM NaCl/0.1% Triton X-100/5mM NaF/0.05 mM EGTA/1mM PMSF). Pellets from cells transfected with vector, pFLAG-β-TRCP, or pFLAG-Cul-1 were vortexed for 10 sec, then incubated on ice for 15 minutes. After centrifugation at 13,000 rpm in an Eppendorf microfuge for 5 minutes at 4^{o}C, the supernatant was added to 20 μl of FLAG M2 beads (Sigma), which were washed with lysis buffer three times before immunoprecipitation. Lysates were incubated with the beads on a rotator for 2 hours at 4^{o}C, followed by one wash with buffer A (25mM Hepes buffer, pH 7.4/0.01% Triton X-100/150mM NaCl) and one wash with buffer B (the same buffer without the Triton X-100). Ubiquitination assay was performed by mixing rabbit E1 (0.2 μg) the E2, Ubch5a (0.8 μg;

from Boston Biochem), ubiquitin (5 μg) or methyl ubiquitin (1.5 μg), Protac (10 μM final concentration unless otherwise specified), recombinant ER (260 ng; from Invitrogen, Inc), and ATP (1mM final concentration) in total reaction volume of 5.0 μl, which was then added to 20 μl (packed volume) of washed FLAG-M2 beads (102). Reactions were incubated for 1 hour at 30°C in a thermomixer (Eppendorf) with intermittent mixing. SDS/PAGE loading buffer was added to terminate the reactions. Western blot analysis was performed by standard methods using polyclonal anti-ER antisera (1:1000 dilution).

Degradation experiments with purified yeast 26S proteasome

Ubiquitination assays were performed as described above. Purified 26S yeast proteasomes (40 μl of 0.5 mg/ml) were added to the ubiquitinated ER on beads and the reaction was supplemented with 6 μl of 1 mM ATP, 2 μl of 0.2M magnesium acetate, and ubiquitin aldehyde 5 μM final concentration as previously described (19, 20). The reaction was incubated for 10 minutes at 30°C with occasional shaking in a thermomixer. For proteasome inhibition studies, purified yeast 26S preparations were preincubated 45 minutes at 30°C with the metal chelators 1,10 phenanthroline or 1, 7 phenanthroline (Sigma) at 1mM final concentration prior to adding to ubiquitinated ER.

Microinjection experiments

293 cells were transfected with a plasmid that expresses GFP-AR (kindly provided by Charles Sawyers, Howard Hughes Medical Institute, UCLA) as described above. Cells were selected with G418 (600 μg/ml) and cultured in MEM with penicillin, streptomycin, and L-glutamine. Prior to experiments, cells were approximately 60% confluent in 6 cm dishes. Protac diluted to 10 mM in KCl (200 mM) with rhodamine dextran (MW 10,000

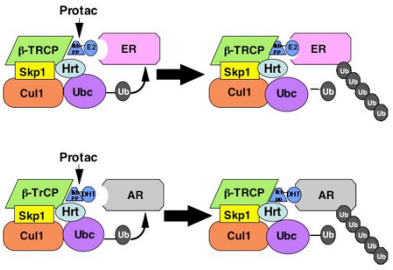
Da; 50 μg/ml) was injected into cells through a microcapillary needle using a pressurized injection system (Picospritzer II). The injected volume was 0.2pl, representing 5-10% of the cell volume. For proteasome inhibition experiments, cells were treated with 10 μM epoxomicin (Calbiochem) for 4 hours or coinjected with epoxomicin (10 μM) and Protac (10 μM). Photographs were taken following injection using a Nikon 35 mm camera. GFP and rhodamine fluorescence were visualized with a Zeiss fluorescent microscope.

Acknowledgements

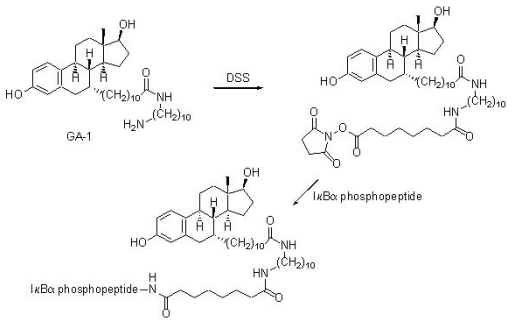
We would like to thank Charles Sawyers for providing the GFP-AR expression plasmid and Eric Davidson (Division of Biology, Caltech) for use of the microinjection equipment and microscope. We are also grateful to Frank Mercurio (Signal Division, Celgene Pharmaceuticals) for his help obtaining GA-1-monosuccinimidyl suberate. This work was supported by the UCLA SPORE in Prostate Cancer Development Research Seed Grant (P50 CA92131 to K.M.S.), CaPCURE (R.J.D., C.M.C., and K.M.S.), Department of Defense (DAMD17-03-1-0220 to K.M.S.), UC BioSTAR Project (01-10232 to K.M.S.), Stein-Oppenheimer Award (K.M.S.), and the Susan G. Komen Breast Cancer Foundation (DISS0201703 to R.J.D.).

Figures

A.


Protac
β-TRCP
ER
Skp1
Hrt
Cul1
Ubc
Ub
Protac
β-TrCP
AR
Skp1
Hrt
Cul1
Ubc
Ub

B.


GA-1
DSS
IκBα phosphopeptide
IκBα phosphopeptide

C.

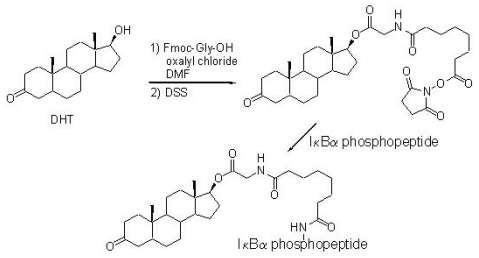

DHT
1) Fmoc-Gly-OH
oxalyl chloride
DMF
2) DSS
IκBα phosphopeptide
IκBα phosphopeptide

Figure IV-1. Protacs to target the ER and AR for ubiquitination and degradation.

(A): Protacs consisting of the IκBα phosphopeptide and either (B): estradiol (E2) or (C): dihydroxytestosterone (DHT) were synthesized to recruit the ER and AR, respectively, to the $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase.

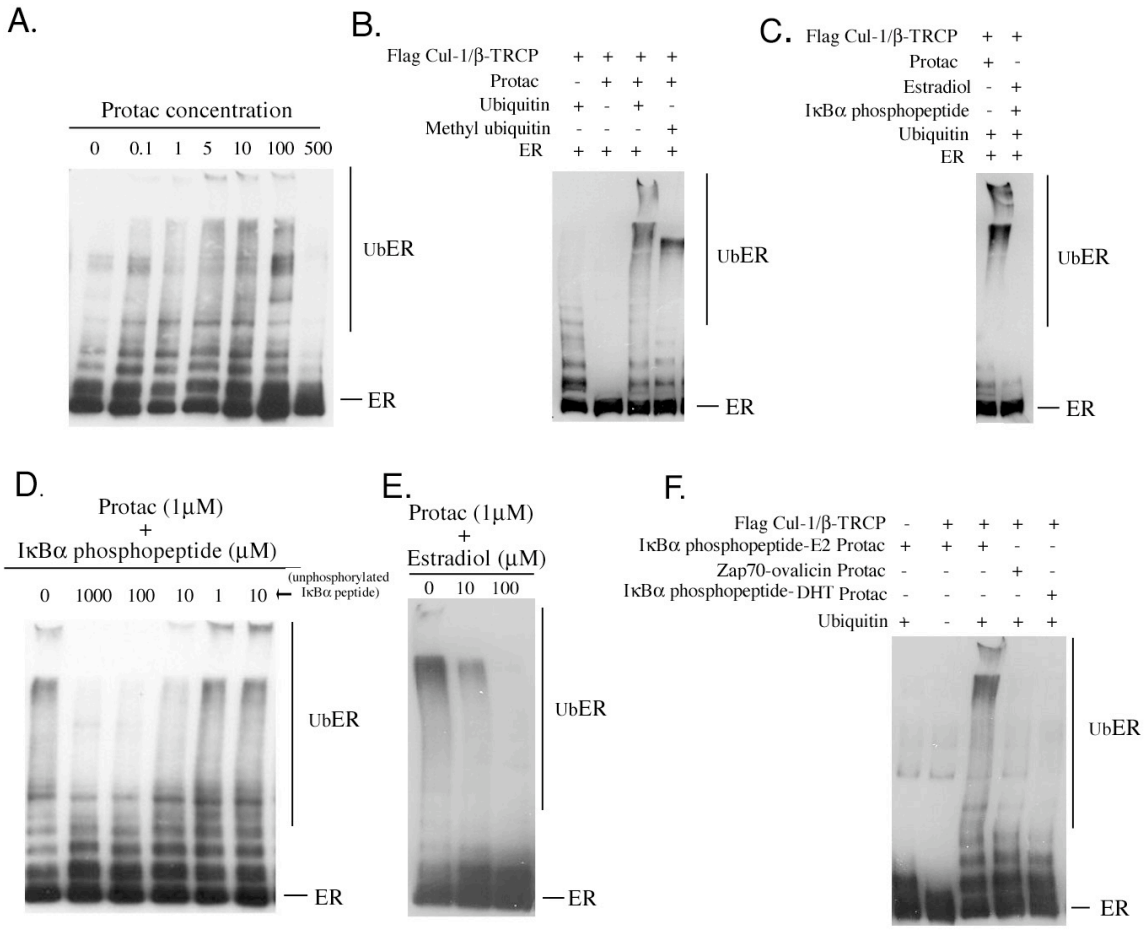

A.
Protac concentration
0 0.1 1 5 10 100 500
UbER
ER
B.
Flag Cul-1/β-TRCP + + + +
Protac - + + +
Ubiquitin + - + -
Methyl ubiquitin - - - +
ER + + + +
UbER
ER
C.
Flag Cul-1/β-TRCP + +
Protac + -
Estradiol - +
IκBα phosphopeptide - +
Ubiquitin + +
ER + +
UbER
ER
D.
Protac (1μM)
+
IκBα phosphopeptide (μM)
0 1000 100 10 1 10
(unphosphorylated IκBα peptide)
UbER
ER
E.
Protac (1μM)
+
Estradiol (μM)
0 10 100
UbER
ER
F.
Flag Cul-1/β-TRCP - + + + +
IκBα phosphopeptide-E2 Protac + + + - -
Zap70-ovalicin Protac - - - + -
IκBα phosphopeptide-DHT Protac - - - - +
Ubiquitin + - + + +
UbER
ER

Figure IV-2. Protac-2 activates ubiquitination of ER *in vitro*. (A): dose-dependent stimulation of ER ubiquitination by Protac-2. Purified ER was incubated with recombinant E1, E2, ATP, ubiquitin, and immobilized $SCF^{\beta\text{-}TRCP}$ isolated from animal cells by virtue of Flag tags on co-transfected Cul1 and β-TRCP. Reactions were supplemented with the indicated concentration of Protac-2, incubated for 60 minutes at 30°C, and monitored by SDS-PAGE followed by immunoblotting with an anti-ER antibody. (B): Protac-2 induces assembly of high molecular weight multiubiquitin chains on ER. Same as (A), except that methyl ubiquitin was added in the place of ubiquitin (lane 4). (C): Estradiol and IκBα phosphopeptide must be covalently linked to promote ER ubiquitination. The reaction was as described in (A), except that IκBα phosphopeptide and estradiol (5μM) were separately added to the ubiquitination reaction instead of Protac-2. (D and E): Free IκBα phosphopeptide (D) and estradiol (E) compete out Protac activity. Same as (A), except that Protac-2 was used at 1 μM. Increasing amounts of IκBα phosphopeptide (lanes 2-5) or 10 μM of IκBα peptide that is unphosphorylated (lane 6, arrow) was added to ubiquitination reaction. (F): Protacs are target-specific. Same as (A), except that zap70-ovalicin and IκBα phosphopeptide-DHT Protacs were used in place of Protac-2, as indicated.

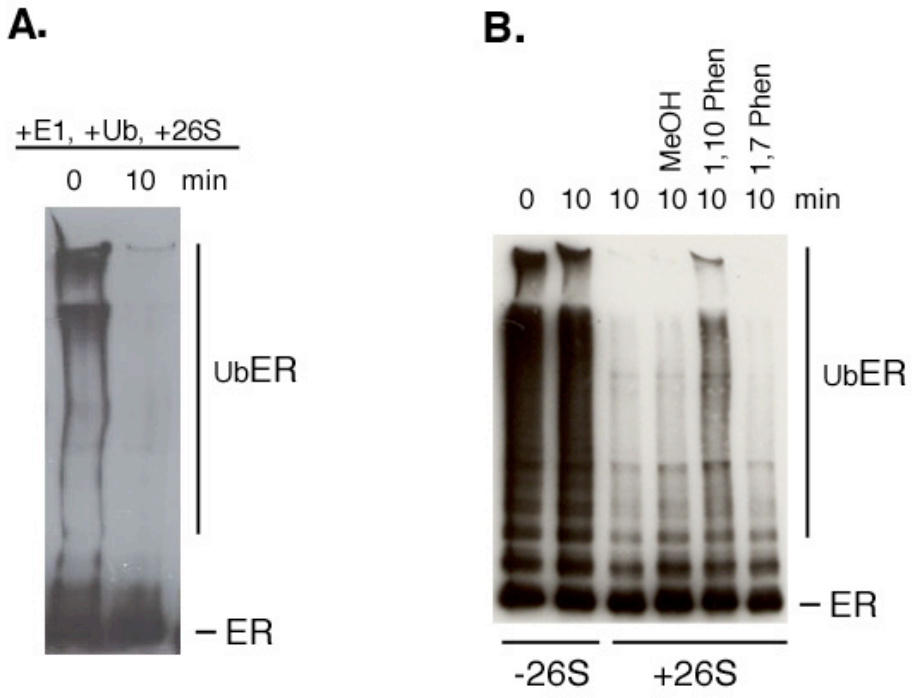
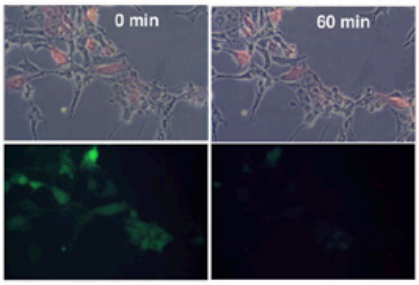
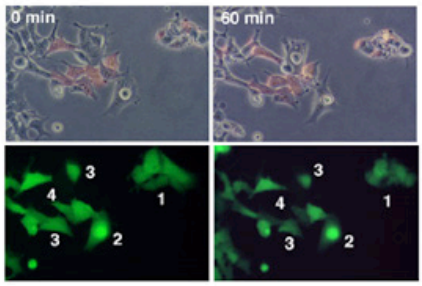


Figure IV-3. Ubiquitinated ER is degraded by the 26S proteasome. (A): Ubiquitination reactions performed as described in the legend to Fig. 2A were supplemented with purified yeast 26S proteasomes. Within 10 minutes, complete degradation of ER was observed. (B): Purified 26S proteasome preparations were preincubated in 1,10 phenanthroline (1 mM) or 1,7 phenanthroline (1 mM) prior to addition. The metal chelator 1,10 phenanthroline inhibits the Rpn11-associated deubiquitinating activity that is required for substrate degradation by the proteasome. Degradation of ER was inhibited by addition of 1,10 phenanthroline, but not the inactive derivative 1,7 phenanthroline.

A.



B.



Degree of GFP-AR Disappearance	Percent (out of >200 cells)
1. NONE	4
2. MINIMAL	16
3. PARTIAL	29
4. COMPLETE	51

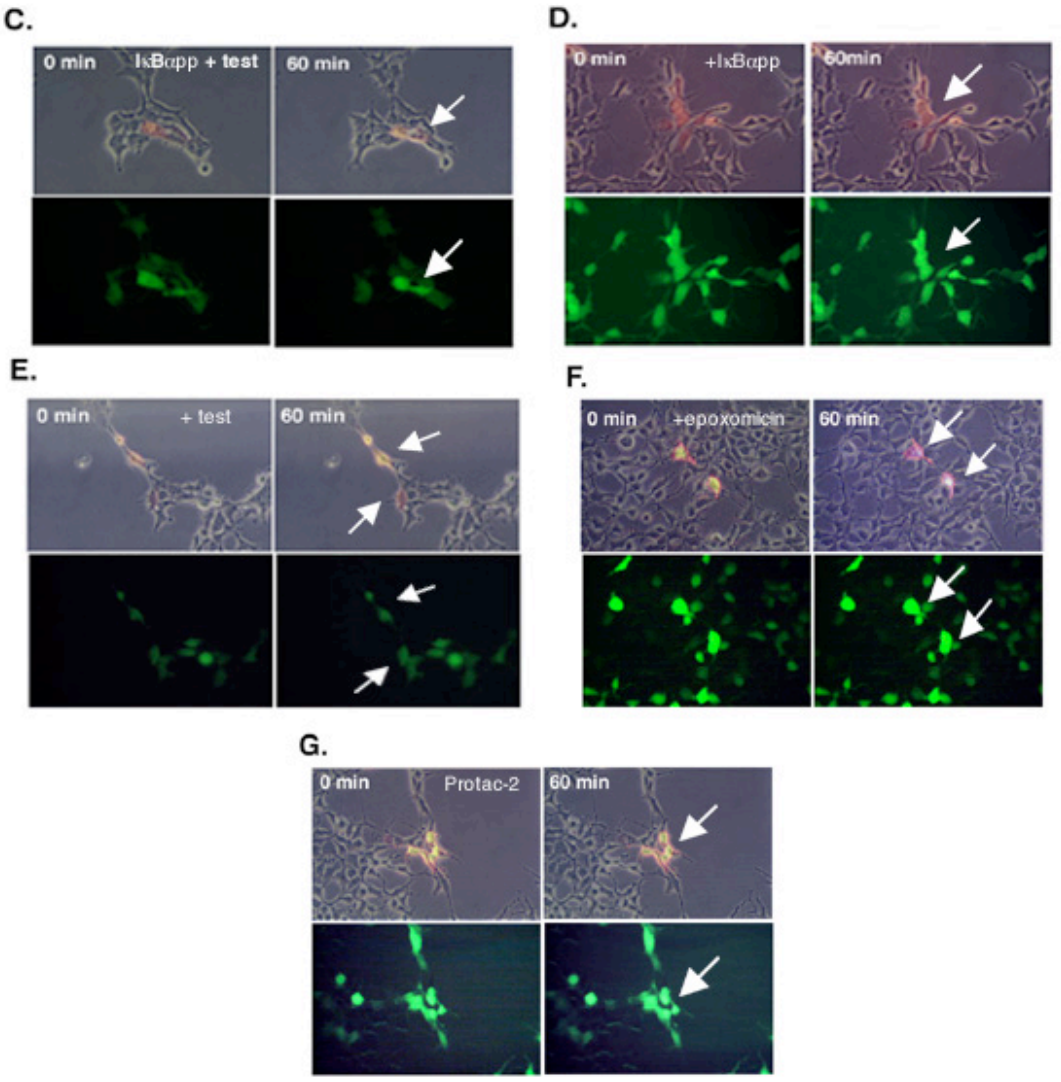

C.
0 min
IκBαpp + test
60 min
D.
0 min
+IκBαpp
60min
E.
0 min
+ test
60 min
F.
0 min
+epoxomicin
60 min
G.
0 min
Protac-2
60 min

Figure IV- 4. Microinjection of Protac leads to GFP-AR degradation in cells. Protac-3 (10 μM in the microinjection needle) was introduced using a Picospritzer II pressurized microinjector into $293^{AR\text{-}GFP}$ cells in a solution containing KCl (200 mM) and rhodamine dextran (50 mg/ml). Approximately 10% of total cell volume was injected. (A): Protac-3 induces GFP-AR disappearance within 60 minutes. The top panels show cell morphology under light microscopy overlaid with images of cells injected with Protac as indicated by rhodamine fluorescence (pink color). The bottom panels show images of GFP fluorescence. By one hour, GFP signal disappeared in almost all microinjected cells. To quantitate these results, we injected over 200 cells and classified the degree of GFP disappearance as being either none (1), minimal (2), partial (3), or complete (4). Examples from each category and the tabulated results are shown in (B). These results were reproducible in three independent experiments performed on separate days with 30 to 50 cells injected per day. (C): Same as (A), except that 293 cells expressing GFP-AR were microinjected with free IκBα phosphopeptide (IκBαpp) plus testosterone (test) (D-F): Same as (A) except that $293^{AR\text{-}GFP}$ cells were microinjected with Protac (10 μM) plus 10-fold molar excess (100 μM) of IκBα phosphopeptide (IκBαpp) (D), testosterone (test) (E), or proteasome inhibitor epoxomicin (10 μM) (F). (G): Same as (A) except that $293^{AR\text{-}GFP}$ cells were microinjected with Protac-2. The controls shown in (C)-(G) confirm that Protac-dependent turnover of AR-GFP depended on intact Protac, and was both saturable and specific.

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CHAPTER V

Targeting the Androgen Receptor for Degradation with a Cell-Permeable Protac

The data presented in this chapter were generated in collaboration with John S. Schneekloth, Jr., Fabiana Fonseca, Michael Koldobskiy, Amit Mandal, and Craig M. Crews. This work was submitted (Schneekloth, et al.; Appendix).

Introduction

Due to the success of the microinjection experiments with the I□B□ phosphopeptide-DHT Protac, we chose to develop a Protac that was capable of entering cells. To this end, our collaborator Craig Crews synthesized a Protac containing the HIF-1 binding peptide ALAPYIP that associates with the ubiquitin ligase, VHL (Von-Hippel-Lindau tumor suppressor (1). This sequence has been shown to be the minimum recognition domain for VHL (2), which serves as the substrate receptor for the ubiquitin ligase complex. Under normoxic conditions, a proline hydroxylase catalyzes the hydroxylation of hypoxia inducible factor 1□ (HIF1□), resulting in recognition and polyubiquitination by VHL. HIF1□ is thus constitutively ubiquitinated and degraded under normoxic condition. In addition to the HIF1□ peptide, an HIV tat poly-D-arginine tag was added on the carboxy terminus to facilitate cell entry and resist nonspecific proteolysis (3, 4, 5). Because a molecule fused to the polyarginine sequence should in principle be cell permeable, the Protac would not need to be microinjected. This HIF-polyarginine-DHT

Protac was tested to potentially develop a novel strategy to inhibit the progression of prostate cancer.

Results

The androgen receptor (AR) has been shown to promote the growth of hormone sensitive prostate tumors. In fact, patients diagnosed with androgen-responsive prostate tumors receive hormonal therapy to control tumor growth, however, these approaches produce significant side effects. There is recent evidence that in androgen-independent prostate tumors, AR can still promote tumor growth. To test the hypothesis that a cell permeable Protac could lead to the degradation of a tumor-promoting protein such as AR, a Protac was synthesized consisting of the HIF-D-Arg peptide linked to the ligand for the androgen receptor, dihydroxytestosterone (DHT).

To determine whether a cell-permeable HIF-DHT Protac could promote degradation of AR, we treated 293 cells stably expressing GFP-AR ($293^{AR\text{-}GFP}$) with increasing concentrations of Protac (Fig. 1A and B). Within one hour, we observed significant decrease in GFP-AR signal in cells treated with 100, 50, and 25 μM Protac, but not DMSO control. Western blot analysis with anti-AR antisera (UBI) verified the downregulation of AR in cells treated with 25μM Protac compared to DMSO control or untreated cells (Fig. 1B, bottom panel). Parental 293 cells do not express GFP-AR and therefore was a negative control. Concentrations lower than 25 μM did not result in GFP-AR degradation (data not shown).

To assess whether Protac-induced degradation was proteasome-dependent, we treated cells with the irreversible proteasome inhibitor, epoxomicin (Fig. 1C, top left panel). Cells were pretreated with 10 μM epoxomicin for 4 hours prior to treatment with 25 μM Protac. Protac-dependent loss of GFP fluorescence was inhibited in cells treated with epoxomicin (Fig. 1C, middle panel) in comparison to cells pretreated with DMSO control followed by Protac (Fig. 1C, right panel). These results demonstrate that the cell permeable HIF-DHT Protac increases the turnover of GFP-AR in cells. Western blot analysis confirmed that decreased GFP-AR fluorescence was due to a decrease in GFP-AR protein (Fig. 1C, bottom panel), which was inhibited when cells were treated with epoxomicin (10 μM).

We next performed experiments to characterize the dependence of GFP-AR degradation on the HIF-D-arg peptide and DHT moieties of Protac. $293^{AR\text{-}GFP}$ cells were treated for one hour with Protac in addition to 10-fold molar excess of testosterone or HIF-D-Arg peptide (Fig. 2d and e). Both testosterone and HIF-D-Arg peptide completely inhibited degradation of GFP-AR by competing with the Protac. Moreover, when added together as separate compounds, HIF-D-Arg and testosterone (25 μM) failed to reproduce the effect of intact HIF-DHT Protac (Fig. 2f). Neither testosterone nor HIF-D-Arg peptide alone had any effect on GFP-AR degradation (Fig. 2g and h). Therefore, these results are consistent with our hypothesis that HIF-DHT Protac acts as a bridging molecule in that one moiety of the Protac, consisting of DHT, associates with the AR, while the other moiety, HIF-D-Arg recruits the AR to the VHL ubiquitin ligase.

To determine whether the HIF-DHT Protac could affect the function of AR, we transiently transfected a plasmid containing two copies of the androgen receptor response region in the rat probasin promoter (ARRE2BP) fused to a luciferase reporter gene (6) (kindly provided by Charles Sawyers) into $293^{AR\text{-}GFP}$ cells. Our results demonstrated that at 4 and 24 hours, there was a 50% decrease in relative luciferase activity in cells treated with Protac (Fig. 3). The difference in activity between Protac treated and DMSO (control) treated cells was statistically significant ($p<0.05$). These experiments were performed in triplicate and on at least two different days. The luciferase activity was normalized using CMV-galactosidase as the internal control. Our preliminary results suggest that not only does cell permeable HIF-DHT Protac lead to degradation of AR in cells, but it also inhibits AR function.

Discussion

These experiments demonstrate that similar to Protac microinjections, a cell permeable Protac can target the AR for degradation in cells. In one sense, Protacs could be used to target any protein within a cell and selectively initiate its degradation, resulting in inhibition of protein function. The advantage of Protacs is that proteolysis is not dependent on the active site inhibition of the target since any unique site of a protein may be targeted, provided there are exposed lysines within proximity for the attachment of ubiquitin. Furthermore, because some E3 ligases are expressed in a tissue specific manner, this also raises the possibility that Protacs could be used as tissue specific drugs.

Future work will focus on the treatment of prostate cancer cells to examine the effects of Protacs on the regulation of endogenous AR, AR function, and cell proliferation. Furthermore, obtaining an efficient drug may depend on a small molecule identified from a chemical library screen that will replace the peptide moiety of Protacs. The goals of future experiments will be to test other Protacs in the context of tumor cells, e.g., breast cancer, followed by preclinical studies in animal models.

In theory, with cell permeable Protacs, one could control a desired cellular phenotype, such as inhibition of a critical transcription factor that is challenging to target pharmaceutically. "Chemical knockout" of a protein could provide an alternative for a genetic knockout, which would be invaluable in the study of protein function. Another potential use of Protacs would be to use diversified libraries to screen for phenotypic effects in a chemical genetic manner. This strategy could be used to identify novel ligands for a target or potentially new protein targets by studying phenotypic changes that result from selective protein degradation. Finally, Protacs could act as drugs to remove toxic or disease-promoting proteins. This strategy may be particularly useful since many diseases, including cancers, are dependent on overexpression of a small number of proteins, including oncogenes. In summary, the expanding potential applications for this technology suggest that Protacs may be widely used in the areas of cell biology, biochemistry, and medicine.

Experimental Procedures

Tissue culture and transfections

293 cells were cultured in DMEM with 10% (vol/vol) FBS (GIBCO), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM). Cells were split 1:5 the day prior to transfection and were 70% confluent in 100-mm dishes on the day of transfection. 293 cells were transfected with 20 mg of GFP-flag-AR expression plasmid (kindly provided by Charles Sawyers, Howard Hughes Medical Institute, UCLA) using calcium phosphate precipitation method. Cells were selected with G418 (600 mg/ml) to establish stable lines. Western blot analysis was performed to document expression of AR (data not shown).

To assess the effects of Protac on AR function, we transiently transfected a plasmid, ARRE2PBLuc, which contains two androgen response regions in the rat probasin promoter from nucleotides –244 to –96 fused to luciferase (6). Twenty micrograms of ARRE2Luc and 1 μg of CMVβ-galactosidase plasmid were added to 0.3 ml of 293 cells stably expressing GFP-AR (resuspended in RPMI and 20% FBS), placed on ice for 5 minutes, and electroporated (200 Volts, 960 capacitance; BioRad Inc.). Cells were resuspended in MEM and plated into 6-well plates with Protac (30 μM) or DMSO control. Cells were harvested at the indicated timepoints and lysed using the Lysis Reporter Buffer (Promega, Inc.). Luciferase and β-galactosidase activity were determined according to standardized methods (Promega, Inc.). Statistical analysis was performed using the JmpIn program.

For GFP-AR degradation experiments, we plated 200 μl of cells at a density of 0.3 or 0.5 X 10^6 cells/ml in 96-well dishes. Protac was reconstituted in DMSO (20 or 10mM stock) to minimize the volume added to cells to 0.5 μl per well for a final concentration of 50 μM. We treated cells with Protac or appropriate controls for one hour. For proteasome inhibition experiments, epoxomicin (10μM final concentration) was added to cells for 4 hours prior to adding Protac.

Western blot analysis-Cells were lysed with boiling SDS-laemmli buffer and boiled for 5 minutes. Equal volume of lysate was loaded onto an 8% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Immunoblotting was performed with blocking buffer (3% milk, 0.1% Triton-X) and rabbit polyclonal anti-AR antisera (1:1000, UBI) or anti-β-tubulin antisera (1:200, Santa Cruz). ECL chemiluminescence was used as the detection method.

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Figures

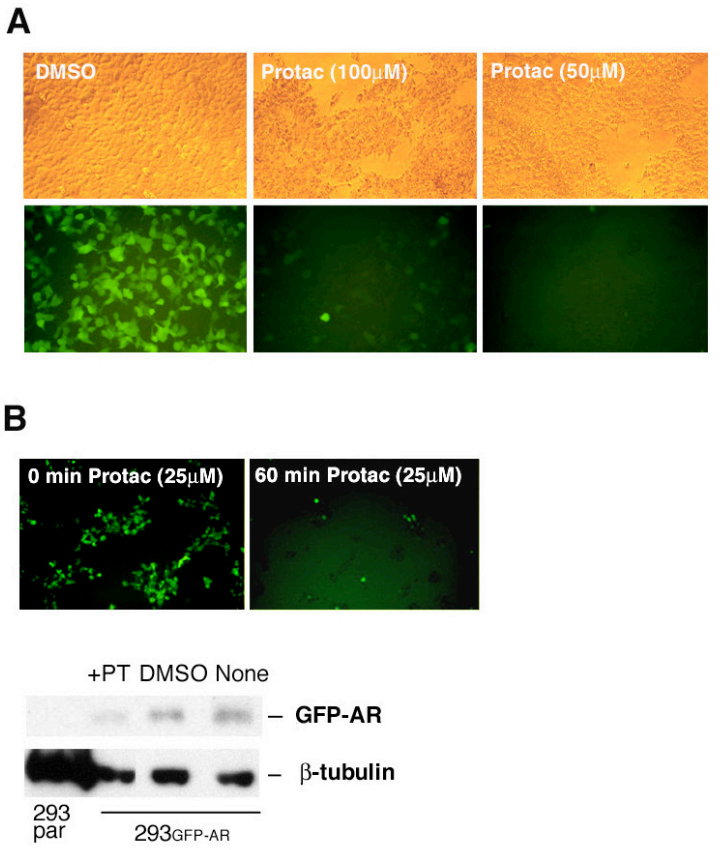
A
DMSO
Protac (100μM)
Protac (50μM)
B
0 min Protac (25μM)
60 min Protac (25μM)
+PT DMSO None
GFP-AR
β-tubulin
293 par
293GFP-AR
C
epoxomicin
epox + Protac (25μM)
Protac (25μM)
DMSO Epox Epox + PT PT
GFP-AR
β-tubulin

Figure V-1. HIF-DHT Protac mediates GFP-AR degradation in a proteasome-dependent manner.

$293^{AR\text{-}GFP}$ cells (0.5 X 10^6 cells/ml) were plated at 50% confluence in a volume of 200 μl of media in a 96-well dish. (A and B): Protac induces GFP-AR disappearance within 60 minutes. Either 100, 50, or 25 μM concentration of Protac or DMSO control in a volume of 0.6 μl was added. Cells were visualized under light (top) or fluorescent (bottom) microscopy one hour after treatment. Photographs were taken with a SC35 type 12, 35 mm camera attached to an Olympus fluorescent inverted microscope. (B): GFP-AR protein is decreased in cells treated with Protac. Lysates were prepared from parental cells (293 par) or GFP-AR expressing cells treated with Protac (+PT), DMSO, or no treatment (None) for 60 minutes. Western blot analysis was performed with rabbit polyclonal anti-AR antisera (1:1000; UBI) or β-tubulin (1:200; Santa Cruz). (C): Epoxomicin inhibits Protac-induced degradation of GFP-AR. Cells were plated at a density of 0.3 X 10^6 cells/ml and treated with 10 μM epoxomicin (Calbiochem) or DMSO for 4 hours prior to adding Protac (25 μM) for 60 min. (D): Western blot analysis was performed with cells in 96-well dishes treated with Protac (25 μM), DMSO (left); epoxomicin (10 μM), epoxomicin (10 μM) + Protac (50 or 25 μM), or Protac alone (50 or 25 μM). These results were reproducible in at least two separate experiments performed in duplicate on different days.

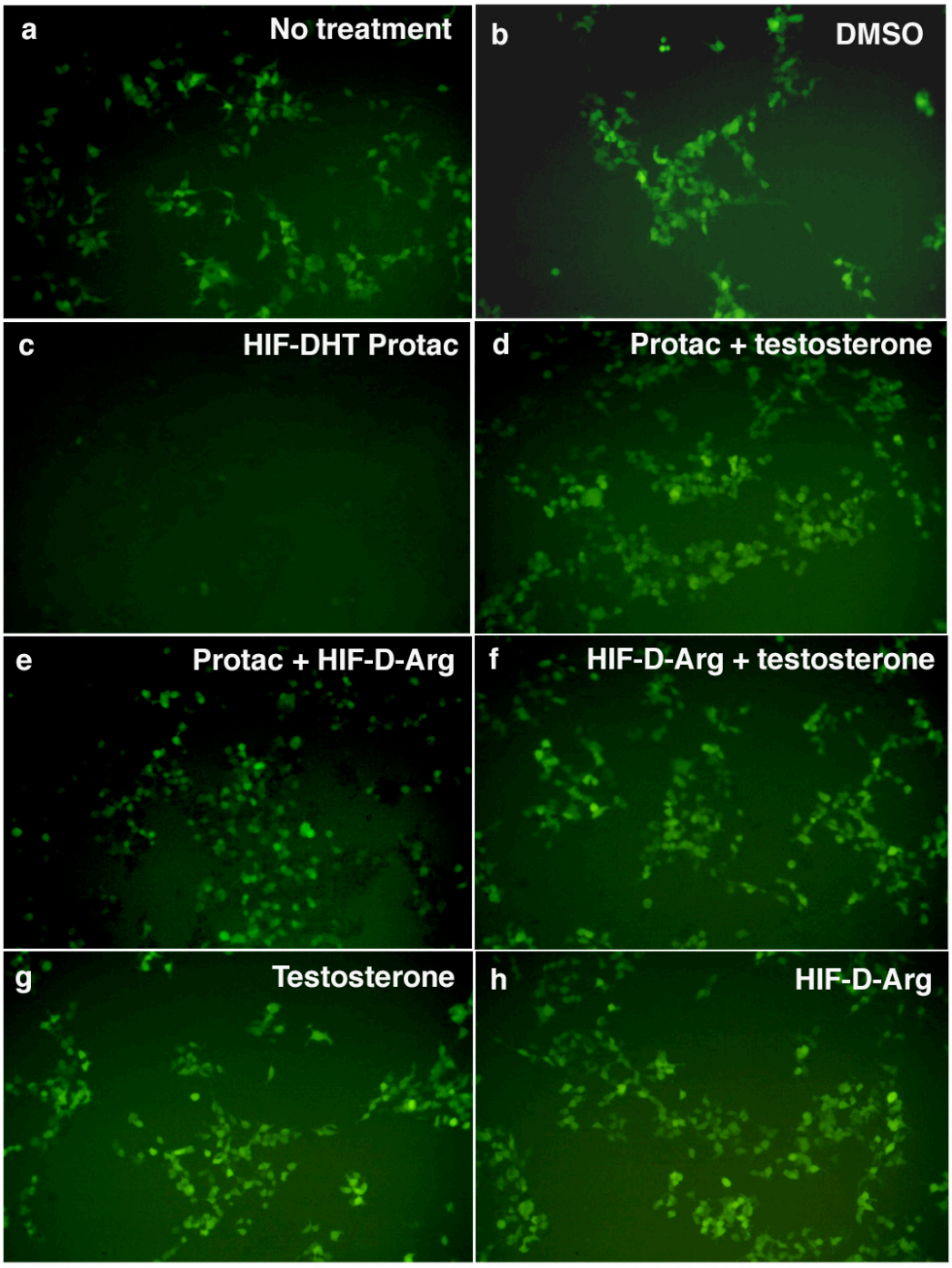
a
No treatment
b
DMSO
c
HIF-DHT Protac
d
Protac + testosterone
e
Protac + HIF-D-Arg
f
HIF-D-Arg + testosterone
g
Testosterone
h
HIF-D-Arg

Figure V-2. The linkage of HIF-D-arg peptide and testosterone is required for HIF-DHT-induced degradation of GFP-AR.

$293^{AR\text{-}GFP}$ cells were plated at a density of 0.3 X 10^6 cells/ml in 96-well dishes containing 200 μl cells/well. Cells were treated with either (a) no treatment, (b) DMSO (equal volume), (c) HIF-DHT Protac (25 μM) alone, (d) Protac + 10-fold molar excess (250 μM) testosterone or (e) Protac + 10-fold molar excess (250 μM) HIF-D-Arg peptide (HIF-D-Arg), (f) HIF-D-Arg peptide (25 μM) + testosterone 25μM together added separately, (g) testosterone (25 μM) alone, or HIF-D-Arg peptide (25 μM) alone. These data are representative of two independent experiments performed on different days.

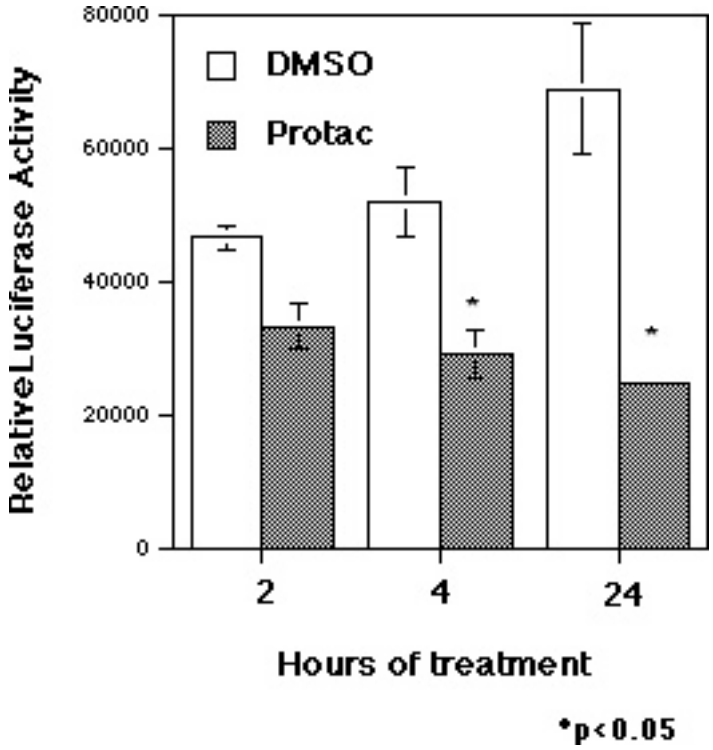

Figure V-3. Protac inhibits ARRE2PBLuciferase activity. 293 cells stably expressing GFP-AR were transiently transfected with a plasmid containing two copies of the AR response region in the rat probasin promoter fused to a luciferase reporter gene. Cells were treated with HIF-DHT Protac (30 □M) or DMSO control at the indicated timepoints. Protac significantly inhibits AR transcriptional activation of ARRE2PBLuc ($p<0.05$). These experiments were performed in triplicate and on at least two different days.

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CHAPTER VI

Future Questions

There are still several questions that will need to be answered before this technology can be considered for therapeutic applications:

1. How can we facilitate entry of Protacs more readily into cancer cells?
2. Does Protac degrade endogenous AR or ER in prostate or breast cancer cells, respectively?
3. Which ubiquitin ligases are optimal to target proteins for ubiquitination and degradation?
4. Which targets would be most amenable to targeted ubiquitination and degradation by Protacs in cancer therapy?
5. What will be the effect on the efficiency of Protacs by replacing the peptide moiety with a small molecule?
6. What are the effects of Protacs on cell proliferation, cell cycle progression, and survival?
7. Are Protacs effective *in vivo*, i.e., mouse models of cancer?
8. Will Protacs be a viable molecular therapeutic approach for the treatment of human disease?