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FOREWORD

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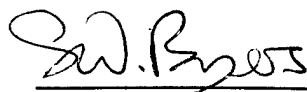
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5. Introduction

Nature of the Problem

Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer (1-5). This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. We now propose an alternative approach in which an oncogenic protein is specifically targeted for intracellular degradation. In order to do this we will take advantage of the permeability properties of the third helix of the antennapedia protein. This will be used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way the ubiquitin-conjugating machinery will be selectively recruited to the target protein which should then be degraded by the proteasome. We will use the cytoplasmic signaling molecule β -catenin as one model system since its oncogenic activity is thought to be regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of β -catenin that increase its protein stability are oncogenic. The β -catenin binding peptide will be based on the region of the tumor suppressor protein APC which constitutively binds β -catenin. A second target will be ErbB-2 a tyrosine kinase strongly associated with breast cancer. The ErbB-2 binding peptide will consist either of the last three armadillo repeats of β -catenin known to constitutively bind ErbB-2 or the SH2 domain of grb2 which can only bind tyrosine phosphorylated ErbB-2. Generally, the work proposed is of great significance to the treatment of breast cancer. More specifically, proof of the principle that direct targeting of oncogenic proteins for intracellular degradation inactivates their transforming capabilities could lead to the development of novel therapeutic strategies based on this approach

Erb-B2:

Erb-B2, located on chromosome 17q21, is a 185 kDa transmembrane receptor-type tyrosine kinase (5, 6). As a member of the EGF family of receptors, it forms homo and heterodimer complexes, which elicit a signal transduction cascade to effect gene expression in the nucleus (6). Upon the binding of its ligands, erb-B2 forms dimers, which cause trans-phosphorylation of tyrosine residues on the cytoplasmic domains of the receptors. The phosphorylated tyrosines act as binding sites for cytoplasmic signaling molecules. This allows different signaling pathways, such as PI3 kinase, ras/MAP kinase, and phospholipase C γ , to be activated. The pathways converge on the nucleus to alter gene expression (6, 7, 8).

Erb-B2 is considered an oncogene because of its role in neoplastic transformations (3, 5). It was originally identified because of its ability to transform NIH 3T3 cells *in vitro* (5). Studies have shown the overexpression of erb-B2 (via gene amplification or overexpression of the gene product, not mutation (2, 4, 9) in gastric, endometrial, salivary gland, ovarian, and breast cancers (10). Since its discovery, some investigators have focused on its role in carcinogenesis of the mammary gland. In the mammary glands of transgenic mice, overexpression of erb-B2 (under the control of the MMTV promoter) leads to the development of mammary tumors and to more rapid regrowth of tumors after chemotherapy (5, 10).

Erb-B2 is overexpressed in 18.5% of stage I and 38% of advanced stage breast cancer patients (4). It is clinically relevant because it has been helpful in predicting overall survival, chances of recurrent disease, stage of disease, number of positive axillary lymph nodes, estrogen receptor/progesterone receptor status, percent S phase, and DNA ploidy (10). In breast cancers, it is associated with poor prognosis and overall survival rate (4, 5). Due to this fact, the oncogene erb-B2 has been the target of many therapies. An example is monoclonal antibodies raised against erb-B2 (Herceptin™) have been shown to inhibit the growth of breast cancer cells that overexpress erb-B2 in cell lines, xenografts, and human patients (11).

The Proposal:

Many therapies have been developed in an effort to treat and cure cancer. One avenue that has been explored is the selective depletion of oncogenes. To date, this has been accomplished by targeting DNA (gene disruption), RNA (ribozymes and antisense), anti-metabolites, and proteins (antibodies) (10-14). These studies have shown that the inactivation or removal of oncogenes or their gene products, such as erb-B2, src, and c-myc, can inhibit tumor growth (8). Another option that may prove more effective or act synergistically with current therapies is the removal of overexpressed or mutant oncogenic proteins by specifically marking them for intracellular degradation. This grant proposes a novel approach to selectively degrading specific proteins of interest for intracellular degradation. Oncogenes will be the proteins of interest, however this system can be applied to any intracellular or membrane bound protein. The basic concept is to create a molecule that will artificially force the colocalization and interaction between a protein of interest and the cells degradation machinery.

The above will be accomplished by creating a chimeric protein that contains three main domains. The first will be responsible for enlisting the activity of a degradation system. This domain will be called the degradation machinery-recruiting domain and will utilize the ubiquitin/proteasome pathway as its method of degradation. The second domain will attract the oncogene of interest, erb-B2, so that it can be targeted for degradation by the first domain. It will be called the erb-B2 attracting domain and will utilize the binding sites for two proteins that interact with erb-B2, namely β -catenin and Grb2. The third domain will function to bring the above peptide across lipid bilayers to deliver it to the cytoplasm of cells and act as a spacer between the degradation machinery domain and the erb-B2 attracting domain. It will be called the antennapedia domain and make use of the lipid permeable third helix of the antennapedia protein.

The Degradation Machinery-Recruiting Domain: Ubiquitin Proteasome Pathway:

The ubiquitin/proteasome pathway is a non-lysosomal proteolytic pathway that degrades proteins by the 26S proteasome complex (16). It accomplishes this task by engaging a series of enzymes to catalyze the transfer of ubiquitin moieties to a specific amino acid on the protein being targeted for degradation (16, 17). In an ATP-dependent manner, the C-terminal glycine residue is attached to a cysteine residue on the enzyme called E1 via a thioester bond. After the activation of this high-energy intermediate, an enzyme called E2 transfers ubiquitin moieties from the E1 to the NH₂ of a lysine residue on the target protein (destruction box). The last enzyme called E3 (which is needed in some situations and in others the E2 is sufficient (17)) acts as a ligase to seal the isopeptide bond between the glycine on ubiquitin and the lysine on the target protein. The process continues except that the activated ubiquitins are attached to a lysine on the ubiquitin that was attached to the target previously. This results in a long chain of ubiquitins attached to the target protein. This long chain of ubiquitins attached to each other signals the 26S proteasome to use proteases to chew up the protein (16) (see figure 2). It is important to note that the E2 and E3 enzymes determine the specificity since they directly interact with the protein targeted for degradation (16).

The above pathway will be used to degrade the protein of interest, erb-B2. The area of a protein where ubiquitins are added and the enzymes interact with is called the destruction box. In previous studies, investigators have reengineered proteins degraded by the ubiquitin/proteasome system so that a protein with a destruction box is connected to another target protein. The target protein has been degraded as long as the destruction box is intact. For example, Brandeis et al. showed that the N-terminal Cyclin B fused with chloramphenicol acetyl transferase (CAT) could cause the removal of CAT protein from NIH 3T3 cells (18). The above suggests that by simply recruiting the ubiquitination machinery by a destruction box, proteins connected to this box can be degraded. This model will be used in our studies except it will be taken a step further. The cyclin B destruction box will be used to recruit the ubiquitination enzymes to degrade erb-B2. But unlike previous studies, our novel approach will hopefully cause the ubiquitination and degradation of a protein interacting with a destruction box chimeric protein but not connected to the destruction box as seen in previous studies. The destruction box of cyclin B is inactive during S phase through G2, causing the accumulation of proteins. From M phase until G1/S,

the destruction box is active and proteins attached are degraded (18). This is important because erb-B2 is tyrosine phosphorylated at this point of the cell cycle, and thus, able to bind some of the proteins it interacts with (eg.= Grb2). Another point of interest is that in the cell, erb-B2 is normally degraded by the ubiquitin/proteasome pathway (15). This is of significance because this proves that erb-B2 can be ubiquitinated and degraded by this pathway.

The Erb-B2 Attracting Domain:

The next logical question is how will the ubiquitin/proteasome enzymes (recruited by the cyclin B destruction box) direct degrade erb-B2? In order to attract erb-B2 to the cyclin B destruction box, the utilization of proteins known to interact with erb-B2 will be used. If the cyclin B destruction box is connected to an erb-B2 binding protein, it is proposed that once in the cell, erb-B2 will bind its partner. The destruction box will recruit the ubiquitination machinery and target the whole construct for degradation.

Two different erb-B2 interacting proteins will be used in the present study in case one is more efficient at binding and aiding in the degrading process of erb-B2 than the other. The first is the cell adhesion/cell signaling molecule β -catenin. Erb-B2 is known to interact with the β -catenin/cadherin cell adhesion complex in cancer cells. Upon stimulation, erb-B2 phosphorylates β -catenin in the cadherin/catenin complex on tyrosine residues. This causes the complex to lose its adhesive strength, leading to increased invasiveness. This suggests that the erb-B2 protein may play a role in regulating the cell adhesion and invasive capacity of cancer cells (19). The last three armadillo repeats of this protein interact with erb-B2 in a tyrosine phosphorylation-independent manner. (This means that the cytoplasmic domain of erb-B2 does not have to be phosphorylated in order to present a binding site for β -catenin) (20, 21).

The other protein is the adapter protein Grb2. Grb2, through its SH2 (src-homology-2) domain, binds phosphorylated tyrosines on the cytoplasmic domain of erb-B2 after trans-phosphorylation of dimerized receptors. It is able, through its SH3 (src-homology-3) domains, to bind to effector molecules in the cytoplasm that eventually transmit a signal to the nucleus through a cascade. This protein acts as a bridge between erb-B2 and effector molecules in the cell. It is a tyrosine phosphorylation-dependent process (22).

Antennapedia Domain:

The next question to be asked is how will the proposed targeting peptide get into cells for therapeutic use? There are several different ways that investigators have used to get peptides into cells (since they do not cross biological membranes freely). For example, a portion of β 2 integrin, myristoylation of amino terminals, and branched peptides (lologomers) have been used; however, they have not proven to be efficacious. The lologomers are highly toxic and myristoylation requires that the protein being imported is localized to the membrane (23). In 1994, Derossi et al. serendipitously discovered that the 3rd helix of the transcription factor antennapedia crosses biological membranes freely. Antennapedia is a homeoprotein involved in many morphological changes and processes mainly in *Drosophila* development (23, 24). The 16 amino acid c-terminal 3rd helix of antennapedia crosses biological membranes in an energy-independent manner and accumulates in the nucleus (25, 26).

Many groups have taken advantage of antennapedia's ability to cross lipid bilayers by creating chimeras consisting of their protein (peptide) of interest linked to the 16 amino acid antennapedia fragment. These experiments show not only that antennapedia chimeras can cross the lipid bilayer, but also the chimeras are stable and do not interfere with the normal intracellular effects of the protein/peptide of interest (26, 27, 28, 29). The 16 amino acid 3rd helix of antennapedia will be used in our studies as a way to deliver the erb-B2 degrading chimera proposed above into cells.

Methods and Results

In the original proposal we proposed to “construct of at least 6 expression vectors containing β -catenin targeting, antennapedia and cyclin B destruction box fusion constructs. These will consist of vectors containing several different β -catenin targeting sequences and cyclin B destruction boxes of various lengths and sequence. Our aim is to ascertain the minimum size of the final trifunctional peptide product that is effective in targeting β -catenin for degradation”. As discussed above we have extended this work to include ErbB-2 as a target as well as β -catenin. Considering the well-documented importance of ErbB-2 in breast cancer we have chosen to concentrate first on this oncogene. During the first year we have successfully made and expressed several recombinant ErbB-2 targeting vectors. Vectors of similar design are also under construction for β -catenin.

Experimental Design:

The primary goal of this project is “Proof of Principle”. We would like to prove that the proposed constructs can specifically degrade erb-B2 and β -catenin and be useful clinically as a therapeutic agents by: 1. showing that the construct can be expressed in cells, 2. showing that it can interact with erb-B2, and 3. showing it can degrade erb-B2. As outlined in the grant proposal the first experiments will be performed using transient transfections of a plasmid/DNA construct (not the actual proposed peptide) into cancer cells. The peptide will be synthesized and used in experiments if it can be shown that transient transfections of the plasmid, that encodes the proposed targeting peptide, can be expressed, bind erb-B2 and degrade it. The proposed construct is composed of several domains, which are listed in Table 1.

Generation of Erb-B2 degrading construct:

Standard recombinant DNA technology was used to construct three different vectors (see figure 1).

Cloning of Cyclin B destruction box: RT-PCR was used to amplify the human cyclin B destruction box from MCF-7 mRNA prepared by standard methods (30). In short, MCF-7 mRNA was incubated with reverse primer (3') (TGG / CTC / AGG / TTC / TGG / CTC / TGG / CAC / T) for 10 minutes at 65 C then 3 minutes on ice. The above mixture was incubated with Reverse Transcriptase obtained from Life Technologies at 37 C for 1 hour. The reaction tube was then placed at 65 °C for 10 minutes and a 3 minute incubation on ice followed. A PCR reaction, with PFU polymerase obtained from Life Technologies, was performed on the above cDNA. The forward primer (5') was added at this time (ATG / GCG / CTC / CGA / GTC / ACC / AGG / A). The cycling parameters are as follows: 94 °C 2 minutes; 94 °C 1.5 minutes, 48 °C 2 minutes, 72 °C 3 minutes---7 cycles; 94 °C 1.5 minutes, 55 °C 2 minutes, 72 °C 3 minutes---23 cycles; 72 °C 7 minutes. The fragment was separated by agarose gel and gel purified (30).

Cloning of Kozak, Flag, Cyclin B, antennapedia into pcDNA3: In order to generate a construct encoding for BamH1 restriction site, kozak, flag, cyclin B, antennapedia, and EcoR1 restriction site, a PCR reaction using Pfu polymerase from Life Technologies was performed (30). The cyclin B gene purified above was amplified using primers containing the sequence for BamH1, kozak and flag on the 5' end and antennapedia and EcoR1 on the 3' end. The primers are as follows:

5' = CGC / GGA / TCC / CCA / GCC / ATG / GAC / TAC / AAA / GAC / GAT / GAC / GAC / AAG / ATG / GCG / CTC / CGA / GTC / ACC / A

3' = CCG / GAA / TTC / CTT / CTT / CCA / CTT / CAT / GCG / CCG / ATT / CGT / GAA / CCA / AAT / CTT / TAT / CTG / GCG / TGG / CTC / AGG / TTC / TGG / CTC / TGG / CAC / T

The cycling parameters used are as follows: 94 °C 2 minutes; 94 °C 1.5 minutes, 70 °C 2 minutes, 72 °C 3 minutes---3 cycles; 94 °C 1.5 minutes, 68 °C 2 minutes, 72 °C 3 minutes---3 cycles; 94 °C 1.5 minutes, 66 °C 2 minutes, 72 °C 3 minutes---18 cycles; 72 °C 7 minutes. The vector pcDNA3, obtained from

Invitrogen, and the construct created above (called BKFCBAE) were cut with the restriction enzymes BamH1 and EcoR1. BKFCBAE was ligated into pcDNA3 BamH1 and EcoR1 sites by using T4 DNA ligase from Life Technologies. The reaction ran at 14 °C overnight (30). A plasmid prep kit from Qiagen was used to make construct DNA and an agarose gel and several restriction enzyme cuts confirmed it was the correct sequence. This construct is now named pcDNA BKFCBAE.

Cloning of β -catenin and Grb2 into pcDNA3 BKFCBAE: PCR amplification of β -catenin and Grb2 was performed as described above for BKFCBAE (30). The primers used were as follows:

β -catenin: 5' = CCG / GAA / TTC / TTT / GTG / GAG / GGG / GTC / CGC / ATG /
GAA

3' = CCG / CTC / GAG / CTC / AGA / CAT / TCG / GAA / CAA / AAC

Grb-2: 5' = CCG / GAA / TTC / TGG / TTT / TTT / GGC / AAA / ATC / CCC / AGA

3' = CCG / CTC / GAG / CGG / CTG / CTG / TGG / CAC / CTG / TTC / TA.

The cycling parameters were the same as for BKFCBAE. Next, the above pcDNA3 BKFCBAE, β -catenin, and Grb2 were all cut with EcoR1 and XhoI and the β -catenin and Grb2 were ligated into pcDNA3 BKFCBAE as described above.

The final constructs: (see figure 1)

Construct #1 = pcDNA3 + BamH1 + Kozak + Flag + Cyclin B destruction box + 3rd helix of Antennapedia + EcoR1 + last 3 armadillo repeats of β -catenin + XhoI

Construct #2 = pcDNA3 + BamH1 + Kozak + Flag + Cyclin B destruction box + 3rd helix of Antennapedia + EcoR1 + SH2 domain of Grb2 + XhoI

Construct #3 = pcDNA3 + BamH1 + Kozak + Flag + Cyclin B destruction box + 3rd helix of Antennapedia + EcoR1

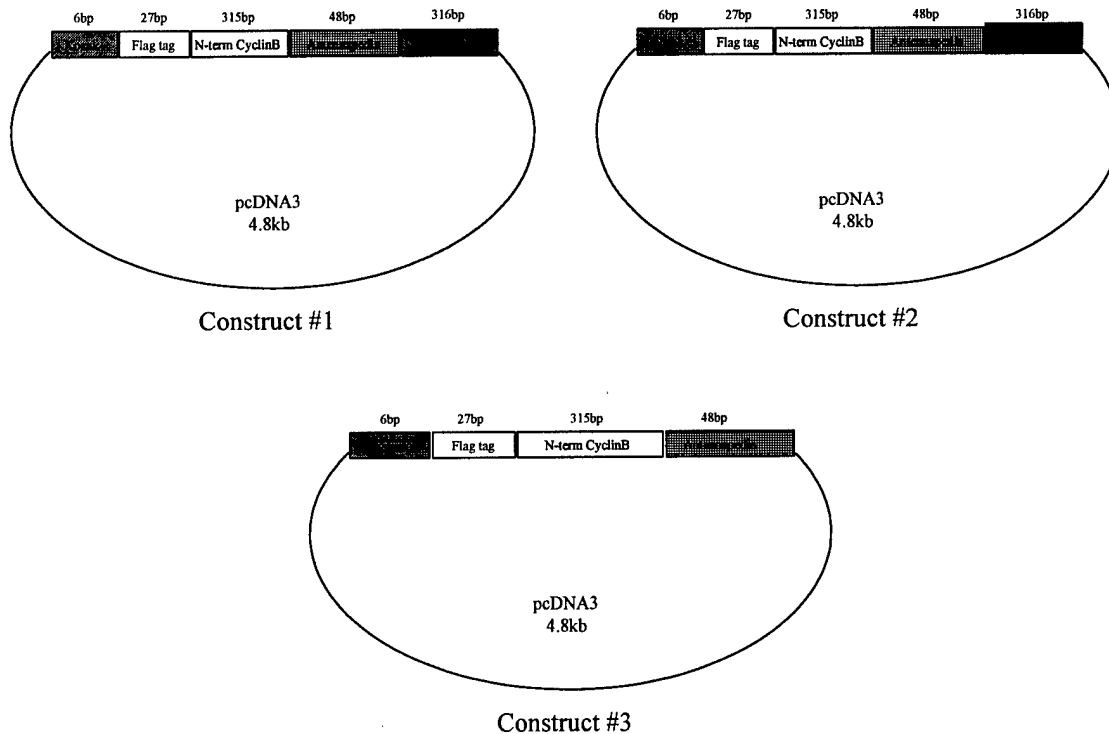
Cell Culture and Transfections: The cell lines used were the MCF-7 breast cancer cell line, the SKOV3 ovarian cancer cell line, and MDA-MB 453 breast cancer cell line. They were chosen because the SKOV3 and MDA-MB 453 cell lines overexpress erb-B2 protein, while MCF-7's have very low levels of erb-B2 protein (31,32,33). The cells were maintained in Dulbecco's Modified Eagles Medium with 10% fetal bovine serum, both obtained from Life Technologies, at 37 °C, under 5% CO₂.

The above cells were used for transient transfection of the three constructs. MDA-MB 453 and MCF-7 cell lines used the calcium phosphate method (30). Briefly, 500,000 cells were plated in 100mm dishes and allowed to grow for 24 hours. For the Ca phosphate method, the DNA was added to CaCl₂ and vortexed well. The above mixture was then added very slowly to hepes buffered saline while vortexing. This was allowed to incubate for 30-45 minutes at room temperature. After 30 minutes, the solution contained a precipitate. The mixture was vortexed well and added slowly to the tissue culture dishes containing the cells of interest. The mixture remained on the cells overnight.

The SKOV3 cell line were transfected using Lipofectamine plus from Life Technologies . For the Lipofectamine plus method, the DNA was incubated with the plus reagent and media for 15 minutes at room temperature, while at the same time the liposomes were incubated with media. The DNA/plus reagent mixture was slowly added to the lipofectamine and incubated for 15 minutes at room temperature. This mixture was slowly added to the cells and remained there for 5 hours. At this point two times the volume of media was added to the cells and allowed to incubate overnight.

For both methods, the cells were washed well 3 times with PBS from Life Technologies and regular media was added back on to the cells. The cells cycled for 24 more hours and the cells were harvested as described below.

Figure 1

Targeting of ErbB2 for Intracellular Degradation

Western Blots: Whole cell lysates were made by washing the cells two times with PBS. Lysis buffer was added to the cells (2% SDS, 60mM Tris pH6.8, 10% glycerol, 5% β -mercaptoethanol, and 0.001% bromophenol blue). They were then scraped and boiled for 20 minutes. Equal amounts of protein were separated by a SDS-PAGE 4-12% gradient gel obtained from NOVEX. The gel was transferred to nitrocellulose (S and S) and a western blot was performed using the anti-Flag M5 antibody from Kodak at a concentration of 5 μ g/mL. After blocking with 5% skim milk, the nitrocellulose blot was incubated with the primary antibody overnight, washed and incubated with secondary antibody for one hour. Then the blot was developed using a chemiluminescence kit from Pierce (30). The expected sizes of the constructs are as follows:

- Construct #1:** 712 bp/3bp per amino acid (110 daltons/amino acid)=26 kDa.
- Construct #2:** 711 bp/3 bp per amino acid (110 daltons/amino acid)=26 kDa.
- Construct #3:** 396 bp/3 bp per amino acid (110 daltons/amino acid)=15 kDa.

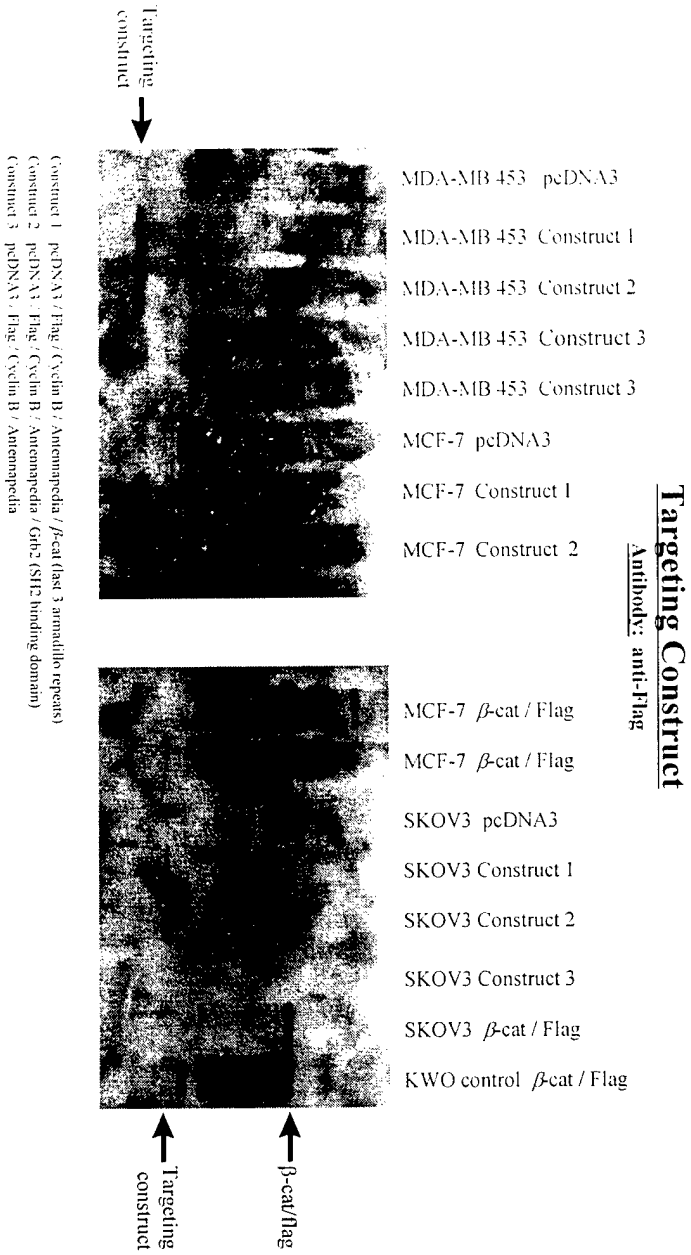
Table 1

Component	Purpose in Construct
pCDNA3	-vector that is constitutively expressed in cultured cells
Kozak	-transcription start site
Flag	-tag that can be detected by antibodies in cells, but cells do not express it naturally, -therefore, when transfected gene is expressed, it can be tracked separately from endogenous protein if has this tag
cyclin B destruction box	-N-terminal destruction box recruits ubiquitination machinery -active in cells after G2 till G1/S transition
amino acid tag	-spacer between recruitment machinery (cyclin B) and target protein (erb-B2) -import up to 100 bp with it across biological membranes -usually nuclear localization but with simple 1 amino acid change, becomes localized to cytoplasm
E-cadherin	-cell adhesion/cell signaling molecule -last 3 armadillo repeats bind erb-B2 -way to bind and aid in degradation of erb-B2 -tyrosine phosphorylation-independent
Grb2	-adaptor protein -SH2 domain binds erb-B2 -way to bind and aid in degradation of erb-B2 -tyrosine phosphorylation-dependent, active when in G1

Results:

The constructs were successfully created and then were transfected into MCF-7 breast cancer cell line, SKOV3 ovarian cancer cell line, and MDA-MB 453 breast cancer cell line. Constructs #2 and 3 were expressed in all of the cell lines, although MCF-7's seemed to have the highest expression level. Construct 3 was not seen in any of the cell lines in this experiment (figure 2). The most likely explanation of this is that the gel was mistakenly run too far so that the 15 kDa marker had run off the gel. This needs to be repeated so that the 15 kDa marker (expected size of C3) is still on the gel.

Figure 2



Key Research Accomplishments

- 1) A number of different targeting vectors have been constructed.
- 2) The targeting constructs have been transfected into three different cells of varying ErbB-2 status
- 3) Two of the constructs yield protein products of the predicted size indicating that the recombinant peptides are stable and can be expressed at relatively high levels.
- 4) The constructs were detected with an antibody directed at the FLAG tag indicating that it is accessible and does not interfere with protein production.

Future Directions and Discussion:

The above results are promising. It seems that the construct can be expressed in cells and the construct is stable. Many other experiments need to be performed to show that erb-B2 is interacting with the expressed targeting construct and being degraded by the ubiquitin/proteasome pathway. Two phases of experiments need to be done sequentially to show "Proof of Principle" and that this degradation concept will be a useful therapy for cancer patients marked with erb-B2 overexpression (see Phases 1 and 2). The first phase consists of more immediate experiments that need to be accomplished to show "Proof of Principle". The second phase of experiments consists of experiments that will prove that the targeting peptide will be a useful therapy.

Potential Problems:

In designing the above experiments and planning this project, a few potential problems arose. First, if these constructs prove to be able to decrease erb-B2 protein levels and are eventually used in animal and clinical trials, it is possible that the constructs will cause an immune response. Another potential problem is that although the ubiquitin/proteasome pathway has been shown to degrade proteins connected to the destruction box, it has never been shown to degrade a protein(s) interacting with a protein that is connected to a destruction box. If it is not successful, it is possible to reengineer an E2 enzyme so that it specifically recognizes erb-B2 (17).

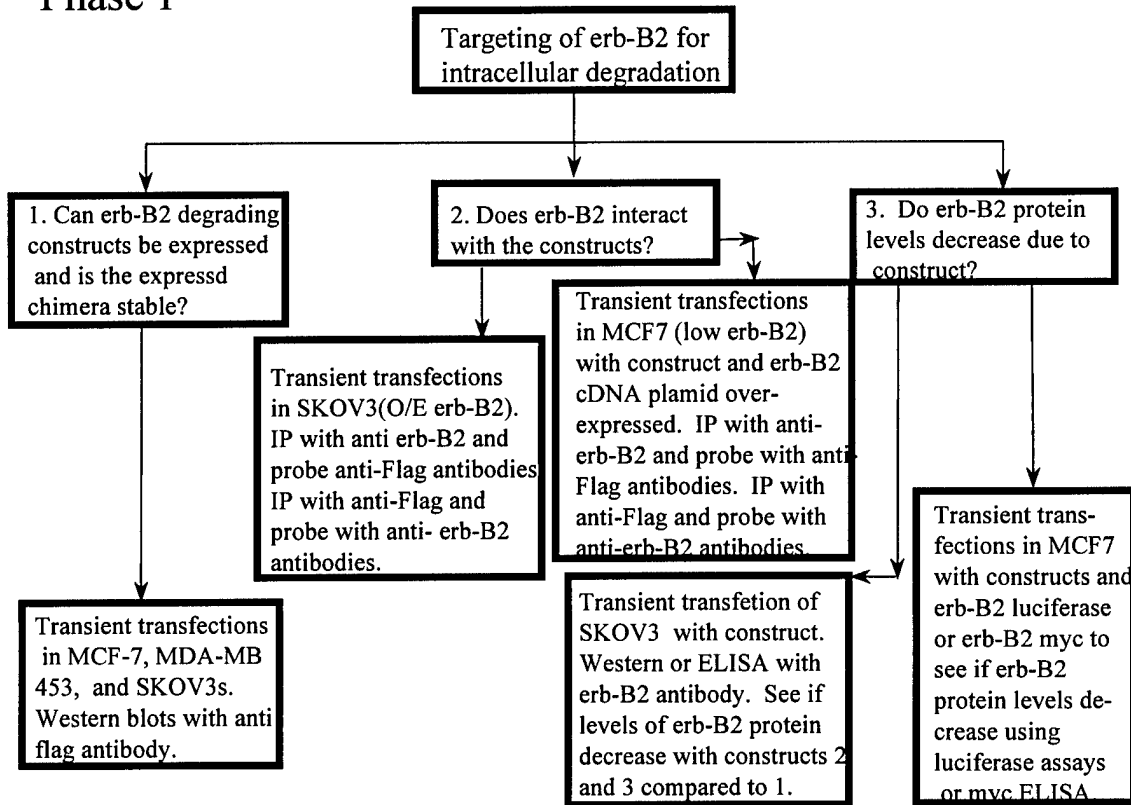
Another concern (which would could actually prove to be beneficial) is if antennapedia has the ability to enter cells by crossing the biological membrane, it can most likely leave the cells too. It could be problematic if the constructs never stayed inside the cells. This could also be helpful because of the "Bystander Effect" (34). One problem with tumor therapies is that it is difficult to target all the cell in tumors. If this construct acts similarly to other therapies and does not reach some cells, with the bystander effect, the cells containing the construct would also be exporting some construct via antennapedia to all adjacent cells. This is a more efficient system that could possibly target tumor cells.

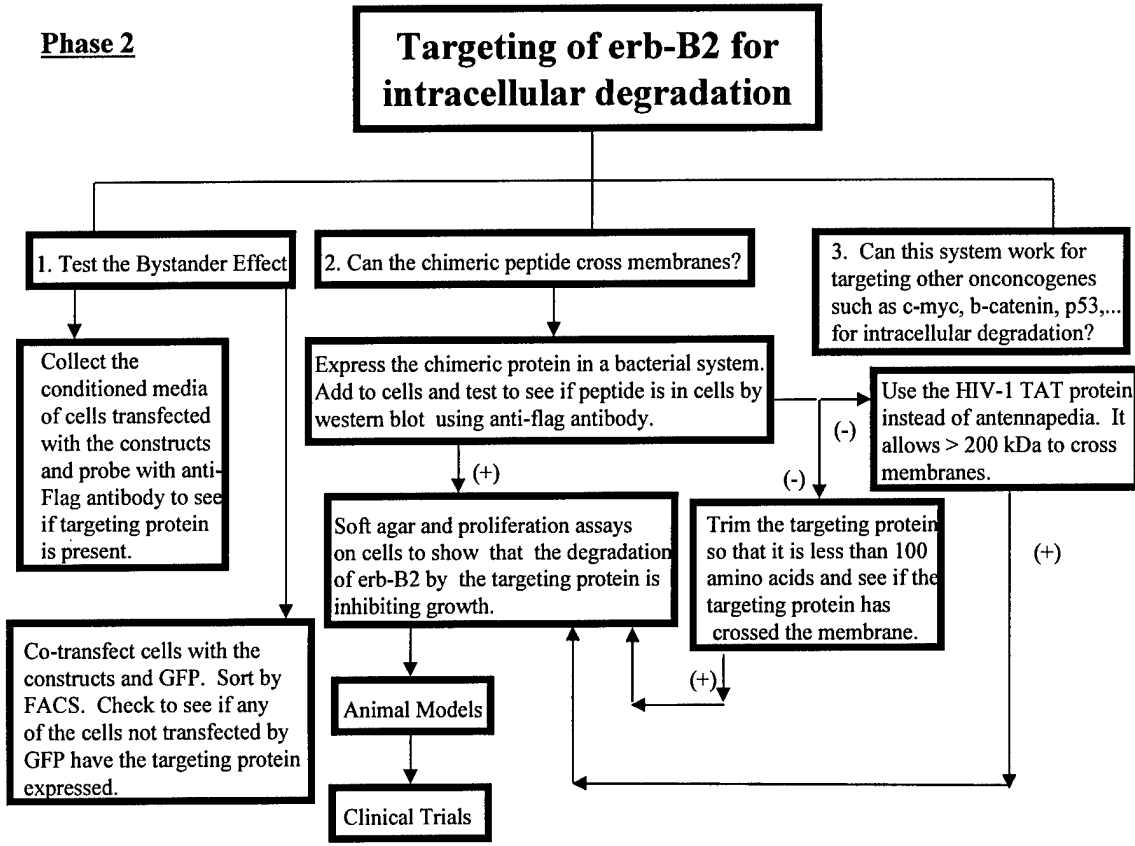
One last potential problem is , it has been shown that antennapedia can only carry 100 amino acids, including itself, across membranes (35). Our chimera is larger than 100 amino acids, making it very likely that it will not cross membranes. There are two possible ways to deal with this problem. One is the proposed chimera can be trimmed so that it is only 100 amino acids long. By determining exactly how many amino acids are needed for binding erb-B2 and recruiting the ubiquitination machinery, the chimera can be decreased in size. Another possible way to deal with this problem is to use the HIV-1 TAT protein instead of antennapedia. Like antennapedia, chimeric TAT proteins can cross biological membranes, but TAT is capable of transporting significantly larger proteins than antennapeida can (36).

Conclusion:

Oncogenes play an important role in the progression of cancer. Their overexpression or hyperactivity contributes to tumorigenesis by promoting uncontrolled proliferation and survival (1, 2). Because of this fact, many oncogenes have been targeted for new cancer therapies. Our work suggests an alternative approach to the common therapies for decreasing the levels of oncogenic proteins in cancerous cells. The targeting of oncogenic proteins for intracellular degradation by the ubiquitin/proteasome pathway may prove to be an effective way of decreasing oncogenic protein levels. The choice of an oncogene in our studies (although any intracellular or transmembrane oncogene should work) is erb-B2 because it is a critical determinant of progression and survival in many cancers, especially breast cancer (3, 5). If this approach successfully causes the degradation of erb-B2 protein, it could conceivably be applied to any cancer with high levels of a defined oncogenic protein as a potential therapy, alone or in combination with existing treatments.

Phase 1





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