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The major advances which have been made encompass work elucidating the structure of the ligand binding domain of the 67 kDa LBP. Phage display studies indicated that three sequence domains in the C-terminal domain of the LBP likely interact with peptide 11. NMR studies of the ligand binding domain of the LBP require that the domain be expressed in a recombinant bacterial system. We have accomplished this for the full length protein and the ligand binding domain. In both cases, the *E. coli* strain expresses large amounts of protein which is readily purified and refolded. Synthesis of candidate 16, which was designed by an early run of the INVENTON program, is essentially complete, and will shortly be investigated for its bioactivity. Refinement of the INVENTON input will soon be undertaken to incorporate new data on the orientation of required amino acid sidechains in peptide 11. Because initial experiments indicated that the 67 kDa LBP could facilitate dimerization of peptide 11 analogs, we compared the bioactivity of peptide 11, a non oxidizable analog of peptide 11 and peptide 11 dimer. The most active species was found to be the dimer, with the Acm protected monomer being the least active.

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FOREWORD

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J. Robert Stuckey Jan 29-99

PI - Signature Date

Table of Contents

Report Documentation Page 2

Foreword 3

Table of Contents 4

Introduction 5

Body of Report 7

Figure 1, Table 1 10

Figure 2, Figure 3, Figure 4 12

Figure 5 13

Figure 6 14

Figure 7 17

Figure 8 20

Table 2, Table 3, Figure 9 22

Conclusions 23

References 25

1

Introduction

Primary tumors can be treated effectively in the short term by surgical removal, often combined with irradiation or chemotherapy, but, this procedure is frequently eventually defeated by metastasis. The 67 kDa high affinity laminin binding protein (LBP) is a cell surface molecule which promotes tumor cell invasion and metastasis. Strong positive correlations of expression of the LBP with poor clinical prognosis have been described for breast cancer and several other solid tumors (1-11). The 67 kDa LBP was originally described as "the laminin receptor", but, with the subsequent description of laminin binding integrin receptors, the apparent common intracellular trafficking (12) and co-overexpression of the LBP and the α6β1 integrin in some solid tumors (13), as well as a demonstrated association of the 67 kDa LBP with the α6β4 integrin (14), it is now considered much more likely that the LBP modulates tumor cell:basement membrane adhesion rather than mediating it. The fact that the LBP is shed from tumor cells in culture in large amounts proportional to the invasive potential of the cells (15,16), and subsequently binds to laminin-1 containing matrix substrates (16), is most consistent with an activity modifying cell:extracellular matrix interactions.

The 67 kDa LBP evolved from the S2 ribosomal class of proteins. A recent study of its evolutionary genomics revealed that a unique evolution of the protein occurred in the C-terminal domain in parallel with the appearance in multicellular organisms of laminin and laminin -like molecules (17). This C-terminal domain has been indicated by us and others as being the ligand binding domain (18-20). The modern protein appears to be multifunctional, having recently acquired its laminin binding activity (17). The working hypothesis is that the evolution of an extracellular matrix binding activity could have occurred via a chaperone function for laminins (17). In spite of the wealth of data supporting the relevance of the 67 kDa laminin binding protein to tumor invasion and metastasis, nothing has been published concerning the mechanism of action of the protein. Such information would greatly enhance our ability to design active mimics of the peptide 11 ligand. Also, while cell surface expression level is strongly correlated with invasive and metastatic ability of tumor cells, until recently, no identifiable mechanism for membrane association of the LBP had been described. Our work (21), recently supported by Butò et al. (22), revealed that the membrane bound form of the protein is acylated with long chain fatty acids, and this is likely to provide a mechanism for membrane association. Furthermore, our studies indicate that the membrane bound form is likely a homodimer of the 32 kDa gene product (21).

A nine amino acid sequence from laminin-1, CDPGYIGSR, known as peptide 11, has been identified as the putative primary binding site for the 67 kDa LBP (23-25). Peptide 11 represents residues 925-933 of the β chain of laminin-1, and a c-loop of an LE (laminin epidermal growth factor -like) repeat in the protein chain. Of importance to potential therapeutic leads, synthetic peptide 11 has been shown to block

invasion of basement membranes by tumor cells (20,26), to reduce experimental tumor lung colonization (metastasis) (20,25) and to inhibit tumor angiogenesis (27). A successful drug based on peptide 11 would slow down or stop the spread of breast cancer, giving the clinician the time needed to effect a cure. Because of their potential as lead compounds for novel anti-metastatic therapeutics, there is a keen interest in producing compounds which mimic peptide 11 in blocking tumor invasion and metastasis. Many groups have synthesized analogs of peptide 11 or of the minimal active sequence, YIGSR, in efforts to improve the biological stability of the peptides (28-32). Only modest increases in activity have resulted, and this is also the case for polymeric and chimeric constructs (33-41). Clearly, accurate mimics of the active conformation of peptide 11 will be needed for effective lead compounds.

The goal of this research project is to design accurate mimics using template -based approaches, and to evaluate their anti-invasive and anti-metastatic activity. If we are successful in synthesizing mimics with good anti-metastatic activity, this will provide a "proof of concept" for our structure -based design approach. The best mimics should then be effective lead compounds suitable for going into combinatorial chemistry programs to provide the most effective derivatives. The combinatorial work would be most appropriate for a commercial company which had an interest in taking the resultant therapeutics through clinical trials.

There are three specific aims which should allow us to accomplish the goal of this research project:

Aim 1 In collaboration with Drs. V. Copié and E. A. Dratz, of the Chemistry and Biochemistry Department at MSU[1], to determine the relevant structural characteristics of the ligand binding domain of the LBP.

Aim 2 In collaboration with Dr. W. Todd Wipke, Professor of Chemistry and Biochemistry, UCSC[2], to undertake structure-based design of non-peptide mimetics for the active conformation of peptide 11 using INVENTON, an artificially intelligent computer program for the design of structural mimetic compounds.

Aim 3 In collaboration with Dr. J. Konopelski, UCSC, to synthesize the most promising structures derived by the INVENTON program, and to evaluate the activities of the new compounds in inhibiting tumor cell invasion *in vitro*, and metastasis in experimental animals.

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[1]MSU = Montana State University

[2]UCSC = University of California, Santa Cruz

2

Body of Report

This is a highly collaborative project with contributions from five different laboratories and two different institutions. It follows that the "statement of work" is complex, and for convenience of the reader, we re-iterate it below.

STATEMENT OF WORK:

Technical Objective (aim) 1

Task 1: Months 1-15: Determine the LBP residues which interact with peptide 11.

Task 2: Months 1-18: Express the ligand binding domain of the LBP in *E. coli*, conduct multidimensional NMR (Nuclear Magnetic Resonance) experiments to determine structural information relevant to drug design.

Task 3: Months 1-15: Provide all relevant information from Tasks 1-2 of this proposal, along with the fully refined bound peptide 11 conformation **(derived from work carried out exclusively on our NIH award),** to Dr. Wipke to improve the peptide 11 template used by INVENTON for the design of peptide mimics.

Technical Objective (aim) 2

Task 4: Months 6-18: Using the artificially intelligent program, INVENTON, design mimics of the LBP-bound conformation of peptide 11 (actually the YIGSR domain from this structure).

Task 5: Months 12-18: Integrate new information coming from tasks 1-3 into the drug design template used by INVENTON.

Task 6: Months 6-18: Evaluate the output structures from INVENTON for potential drug lead compounds. Synthesize the most approachable of these.

Task 7: Months 6-18: Work with Dr. Wipke's group in providing heuristic rules for determining the relative ease of synthesis for output structures from INVENTON.

Task 8: Months 12-22: Provide Dr. Starkey's group with mimetic compounds for limited preclinical tests.

Technical Objective (aim) 3

Task 9: Months 1-12: Test informative analogs of peptide 11 for anti-invasive and anti-metastatic activity.

Task 10: Months 12-24: Test mimetic compounds for 1) tissue culture toxicity, 2) anti-invasive activity and 3) anti-metastatic activity.

Progress on Technical Objective 1, task 1.

Experimental Methods and Procedures

The methodology originally outlined in the grant application was restricted to mapping the contact residues in the LBP by using our photo-crosslinking biotinylated analog of peptide 11 (16). The derivatized LBP and its cleavage fragments would be isolated using monovalent avidin matrices, and final analysis would be carried out using mass spectrometry sequencing techniques. The proposed methodology for isolating derivatized LBP and cleavage products relied on the use of one of the two commercially available monovalent avidin matrices. Unfortunately, both gave very poor yields which we think is due to the methods used to reduce the valency of the avidin. Most likely this is done via controlled alkylation, and the added chemical groups could easily result in stearic problems when attempting to bind a relatively large biotinylated substrate. Commercial anti-biotin antibodies were also found to be of little use, in this case due to their low affinity. We, therefore, decided to make our own rabbit antibodies for this work. Appropriate antibody specificity was sought using immunizing antigens consisting of biotin and biotinylated peptide 11 crosslinked to an irrelevant protein (KLH - keyhole limpet hemocyanin). After removal of KLH specific responses, we tested the antibodies by Western blot analysis. Good titers were found to biotinylated proteins and derivatized LBP. Assuming that we get good yields of isolated, derivatized LBP using these new antibodies, then mass spectrometry analysis should be underway within the next 6 months.

Because difficulties were found with the initial isolation steps needed for mapping LBP contact residues using the photo-crosslinking peptide 11 analog, we carried out a series of experiments using phage display mapping to identify sequences within the LBP which interact with peptide 11. This alternative experimental approach cannot provide the detail to identify the actual contact residues, but, nonetheless, can provide very useful preliminary data identifying the interacting sequences. The J404 9-mer random phage display library (42) was used to identify sequences which bind to laminin-1 and elute with heparan sulfate or peptide 11. Laminin-1 derivatized plates were used for biopanning. Three consecutive rounds of low pH elutions were carried out, followed by three rounds of specific elutions,

each consisting of a heparan sulfate elution followed by a peptide 11 (CDPGYIGSR) elution. The random sequence inserts were sequenced for phage populations eluted at low pH, by heparan sulfate and by peptide 11. The phage insert sequences were then compared to the sequence of the LBP (Figure 1) looking for similarities which would identify regions of the LBP which could bind to peptide 11. Heparan sulphate elution was included as previous work has suggested that heparin mediated binding of the LBP to laminin-1 could occur in some cases (43,44).

Assumptions

We anticipated that results from the phage display experiments would identify sequences within the LBP which contain the contact residues for peptide 11.

Results

Specifically eluted phage populations exhibited three classes of mimitopes for different regions in the cDNA derived amino acid sequence of the 67 kDa laminin binding protein (LBP) (Figure 1). These regions were 1) a palindromic sequence known as peptide G, 2) a predicted helical domain corresponding to LBP residues 205-229, and 3) TEDWS -containing C-terminal repeats (Table 1). All elution conditions also yielded phage with putative heparin binding sequences (Table 1). We modeled the $LBP^{205-229}$ domain, which we demonstrated by circular dichroism (CD) to have a helical secondary structure, and determined that this region likely possesses heparin binding characteristics located to one side of the helix, while the opposite side may contain a hydrophobic patch where peptide 11 could bind. Using Elisa plate assays, we demonstrated that peptide 11 and heparan sulfate both individually bound to synthetic $LBP^{205-229}$ peptide. We also demonstrated that synthetic PATEDWSA peptide could inhibit tumor cell adhesion to laminin-1. These data support the proposal that the 67 kDa LBP can bind the β-1 laminin chain at the peptide 11 region, and suggest that heparan sulfate is a likely alternate ligand for the binding interactions. Our results also confirm previous data (18) suggesting that the most C-terminal region of the LBP, which contains the TEDWS repeats, is involved in cell adhesion to laminin-1, and we specifically indicated the repeat sequence in that activity.

Discussion and Recommendations

These experiments indicate that peptide 11 interacts with three different sequences domains in the 67 kDa LBP. The data also suggests that heparin/heparan sulfate is an alternate ligand for the 67 kDa LBP. As expected, the ligand binding domain of the LBP appears to be quite complex. Any structures which we develop for the active conformation of peptide 11, and for the ligand binding domain of the LBP, should be compatible with these data on peptide 11 interacting sequences.

Progress on Technical Objective 1, task 2.

Experimental Methods and Procedures

Expression of the ligand binding domain of the LBP in *E. coli*: This work is being carried out in collaboration with Dr. Valérie Copié at Montana State University. The first step in conducting the NMR structural studies of the ligand binding domain of the LBP is to express this domain in bacteria. This will allow for heavy isotope labeling of the domain at reasonable cost. The coding region for the full length expression product was obtained from our mammalian vector and cloned into the pTrcHis B prokaryotic expression vector (Invitrogen). Top 10 *E. coli* cells (Invitrogen) were successfully transformed, and they

67 kDa Laminin Binding Protein

```
1    MSGALDVLQM KEEDVLKFLA AGTHLGGTNL DFQMEQYIYK RKSDGIYIIN
51   LKRTWEKLLL AARAIVAIEN PADVSVISSR NTGQRAVLKF AAATGATPIA
101  GRFTPGTFTN QIQAAFREPR LLVVTDPRAD HQPLTEASYV NLPTIALCNT
151  DSPLRYVDIA IPCNNKGAHS VGLMWWMLAR EVLRMRGTIS REHPWEVMPD
201  LYFYRDPEEI EKEEQAAAEK AVTKEEFQGE WTAPAPEFTA TQPEVADWSE
251  GVQVPSVPIQ QFPTEDWSAQ PATEDWSAAP TAQATEWVGA TTDWS
```

Figure 1. Sequence of the 67 kDa LBP (monomer). Cys residues are indicated with an asterisk, peptide G sequence by a double underline, 205-229 helical region by a single underline, and one DWS containing repeat is shown with a dotted underline.

Sequence homology with:	Sequences eluted by peptide 11	Sequences eluted by heparin/heparan sulfate
LBP[205-229]	RDPEEIEKEEQAAAEKAVTKEEFQG GMKAVR IQG GKAMLDRAS	RDPEEIEK EEQAAAEKAVTKEEFQG DRTAMQVAA DRTAMQVAA VVSKISEAG GGSVAFRAG
LBP - Peptide G	IPCNNKGAHSVGLMWWMLAREVLRMR KPWWRTNTA WHRTMWWWP PWWWMTRHW (rev)	IPCNNKGAHSVGLMWWMLAREVLRMR GPGAWWGSA
LBP - C terminal repeats	QPATEDWS QN TD WLGNL	QPATEDWS None
Heparin binding sequences	HARSHYPWY KWKWPDRPK SLEHRAFRN VPFYSHSKL GKLNLGGYK KMNKGVVNP	SKMHRNSWF AKIPAGRDR VPFYSHSKV KMNKGVVNP

Table 1. Mimitope sequences present in phage specifically eluted from laminin-1.

produced a protein of the correct molecular weight which stained positively with our anti-LBP antibody in a Western blot. However, the Trp promotor in this vector is not particularly efficient, and only modest yields of the LBP were obtained.

A second vector was then tried. This is the pET-30 vector from Novagen which utilizes the more efficient T7 promotor system. This time we used CD41 *E. coli* cells which have been successfully used in our hands to produce isotopically labeled peptides. On induction with IPTG (Isopropyl-β-D-thiogalactopyranoside), large quantities of the expressed LBP were obtained (Figure 2), and the same was the case for the C135 LBP ligand binding domain. We utilized the N-terminal poly-His tag for isolation of the expressed recombinant protein by Ni affinity (Figure 3) The pET-30 vector contains two N-terminal protease cleavage sites for removing the poly-His tag. Closest to the expressed protein sequence is an enterokinase site. We abandoned this site when we could not achieve cleavage efficiencies greater than 50%. The more distant thrombin cleavage site worked very well with close to 100% efficiency (Figure 4). Biotinylated thrombin is used for the cleavage, and it is easily removed from the expressed protein preparation over a streptavidin column. Free poly-His sequence is removed by a second pass over the Ni column. It is clear that the pET-30 vector system would work well for our needs. A final refinement, now being attempted, is to utilize a modification of this vector, pET-15b which does not contain an enterokinase cleavage site, and which has the thrombin cleavage site immediately N-terminal to the expressed protein sequence. This vector would provide expressed protein without any additional irrelevant sequence from the vector construct.

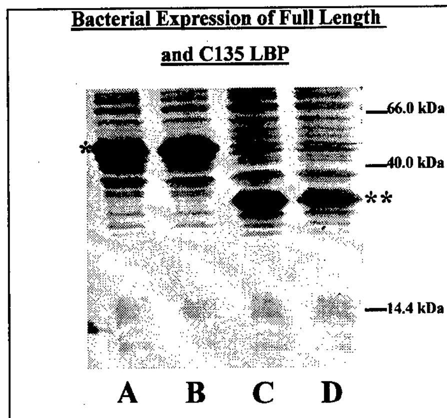
The molecular weight of the isolated expressed C135 ligand binding domain was checked by time of flight MALDI (matrix assisted laser desorption) mass spectrometry, and the success of refolding the domain by circular dichroism (CD) spectroscopy.

Assumptions

The only assumption being made for this task is that the bacterial monomeric protein will function like the high molecular weight form of the protein. The only published data indicate that, for binding to laminin-1, this is true (45). Unsupported statements (no data given) in the literature question this finding (46). Therefore, we will need to conduct a series of control binding experiments with the monomer. So far, our preliminary experiments indicate that the full length bacterial product interacts with peptide 11 in the same way as the high molecular weight mammalian protein does.

Results

As discussed in the "Methods" section, we have succeeded in generating efficient bacterial expression systems for both the full length LBP and the C135 ligand binding domain (Figure 2). The expressed proteins are readily purified over Ni columns using imidazole elution (Figure 3), and the poly-His sequence is efficiently removed using thrombin (Figure 4). After refolding the initially denatured bacterial expression product, the CD spectrum shows no evidence of random coil structure indicating that the protein has refolded well (Figure 5, Panel B). The LBP CD spectrum is dominated by an alpha helical profile (Figure 5, Panel A). Since the majority of the predicted alpha helix is in the C-terminal domain of the protein, we expect a largely alpha helical profile for the C135 LBP domain also. The spectrum shown in Figure 5, panel B shows considerable distortion at the shortest wavelengths, so better spectra need to be collected to assess the probable % alpha helix present. Figure 6 shows the mass spectra obtained for the isolated C135 LBP domain. Figure 6, panel A shows the domain before removing the poly-His sequence, and panel B after this has been removed using thrombin cleavage. In both cases the mass is extremely



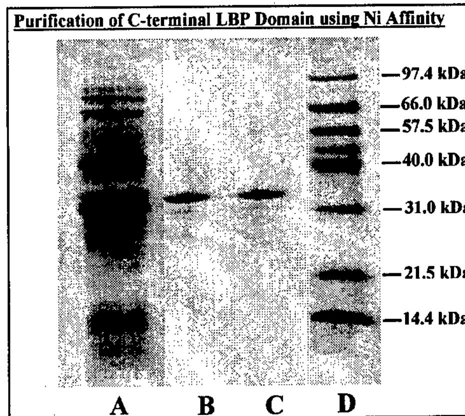


Figure 2. Whole bacterial cell lysates resolved on Coomassie Blue stained SDS-PAGE[1] gels. Single asterisk indicates the position of the full length LBP expression product and the double asterisk that of the C135 LBP expression product.

Figure 3. Purification of C135 LBP expression product using Ni affinity column. Resolved on Coomassie Blue stained SDS-PAGE gel. Lane A = whole cell lysate, lanes B and C = imidazole eluate from Ni column, lane D = molecular weight markers.

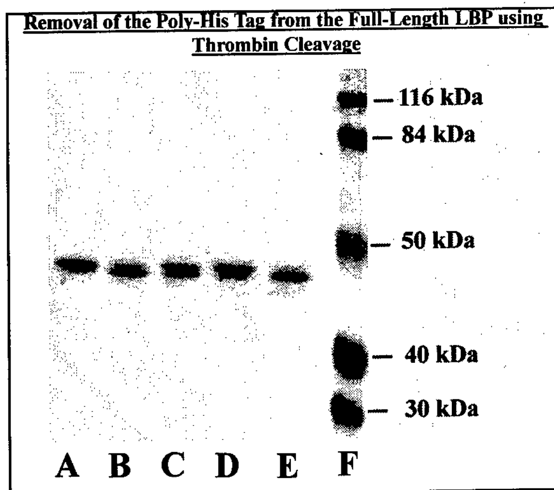
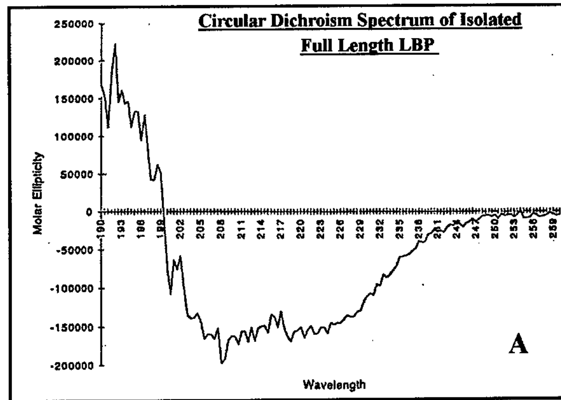


Figure 4. Full length LBP bacterial expression product resolved on Coomassie Blue stained SDS-PAGE gel. Lane A = uncut product, lanes B, C, and D = partially thrombin cut product, lane E = fully thrombin cut product, lane F = molecular weight markers.

[1] SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.



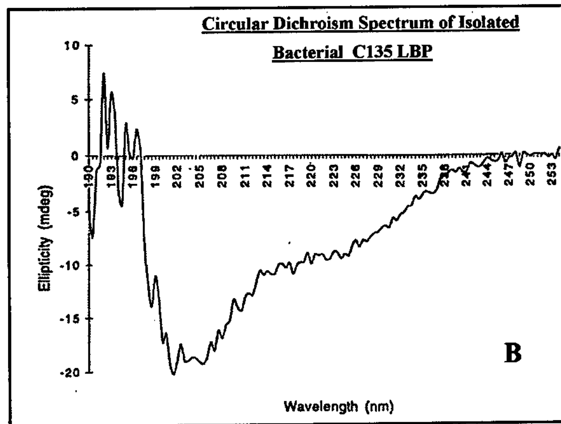


Figure 5. Circular Dichroism Spectra for (A) mammalian isolated 67 kDa Laminin Binding Protein, and (B) recombinant bacterial expressed C135 Laminin Binding Protein domain.

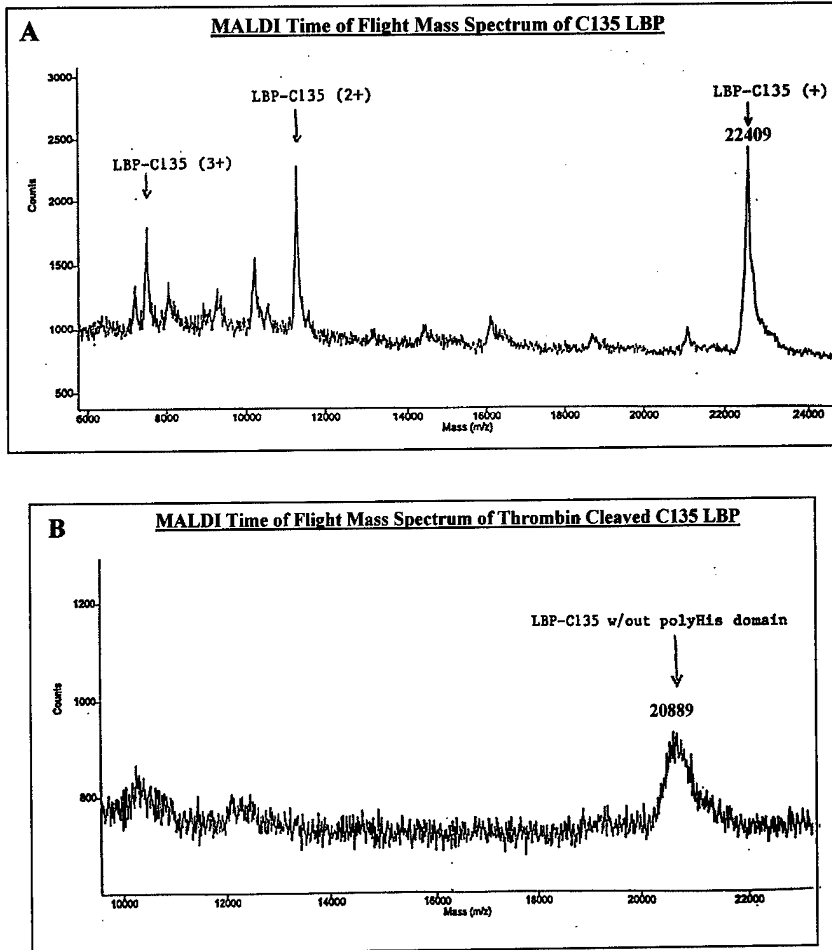


Figure 6. Mass Spectrometry analysis of the C135 LBP domain. Panel A = isolated expression product prior to cleavage with thrombin. Panel B = isolated expression product after the poly-His sequence was cleaved off with thrombin.

close to that calculated (22409 for the domain and 20889 after cleavage with thrombin).

Discussion and Recommendations

The current bacterial expression system is working well, and could be used to provide heavy isotope labeled C135 LBP product for the NMR experiments. If the modified pET vector, pET-15b, is as efficient, we will substitute it, as it is considered that the presence of the least amount of irrelevant sequence is the best situation for the future NMR experiments. We will know this within a month from now.

Progress on Technical Objective 1, task 3.

Experimental Methods and Procedures

Although structural work on peptide 11 is exclusively supported by our NIH award, the data it provides is key to this Army project. Therefore, a brief update of the most relevant recent findings are provided here.

Information about the orientation of required animo acid sidechains in the peptide 11 template is critical to the design of active peptido-mimetics. Unfortunately, there are very few NMR crosspeaks to the required Tyr, Ile or Arg sidechains in aqueous solution. We took advantage of the fact that the relatively viscous DMSO (Dimethyl Sulfoxide) solvent would slow down the motion of the peptide and allow for better definition of individual structure conformations. The two-dimensional NMR spectra run in DMSO provided a greatly increased number of crosspeaks. For the first time, a useful number of sidechain cross peaks were found. The new sidechain crosspeaks support a close relationship between the Ile and the Tyr sidechains as well as providing information on the position of the Arg sidechain.

Assumptions

None

Results

The new structural information about the orientation of the required amino acid sidechains in peptide 11 will be supplied to Dr. Wipke in the next couple of months. This will allow him to make important adjustments to the input of the INVENTON program. Currently, because of a lack of this type of information, we have restricted the freedom of these sidechains to that expected in any flexible linear small peptide. After the input parameters have been adjusted, the generation of new structures from INVENTON should be considerably improved in terms of their likely anti-metastatic activity.

We do not yet have any detailed structural data on the ligand binding domain of the LBP which would enable Dr. Wipke to make additional adjustments to the INVENTON program input. These data should start to become available towards the end of this next year. It should be noted that provision of structural data to Dr. Wipke is an ongoing process and will happen throughout this project.

Discussion and Recommendations

Our new information on required sidechain orientations in peptide 11 is so important to drug design

that it is considered well worth while to carry out additional INVENTON runs as soon as the data is incorporated into the program. We anticipate the generation of more constrained mimetic structures from these new runs. Dr. Konopelski will evaluate their ease of synthesis, and initiate actual synthesis on one or two of the most approachable ones.

Progress on Technical Objective 2, tasks 4 and 5.

Experimental Methods and Procedures

Structural information from the full peptide 11 sequence is being derived because the minimal active domain, YIGSR, is too small to provide sufficient NMR crosspeaks. However, only the structure coordinates etc. for the YIGSR region are used in the drug design process. This provides a reasonably sized template for the design of non-peptide mimics. Design of potential mimics is carried out using the artificially intelligent program, INVENTON. The pharmacophore hypothesis provides the computer program with information as to what aspects of the peptide were likely important in recognition and binding with the LBP. The receptor (LBP) sees the shape of the peptide, and interacts with its electron density and dipolar nature. For template-based design, INVENTON uses the field of the template. Clearly, candidate structures must have chemically stable functionality. They must also survive the human digestive system to allow for oral therapy.

Now that we are working to obtain an NMR structure defining the ligand binding domain of the LBP, we will also be able to utilize characteristics of the LBP binding pocket in mimetic design. This was the original way INVENTON was used. The computer algorithms design specific mimics for the detailed bound structure completely automatically using FASM, fragment assembly for construction. After all structures are ranked, individual candidates are examined and molecular dynamics simulations are performed to evaluate flexibility and the ability of the molecule to retain the desired conformation. Dr. Konopelski is responsible for evaluating the probable ease of synthesis of mimetics, and for choosing the actual synthetic approaches.

Assumptions

The main assumption in this part of the work is that the program INVENTON will design mimics which are at least as good as those designed by a human chemist. Our experience with the program working on other projects is that this is the case. Furthermore, the program is far more innovative, producing structures pharmaceutical chemists would not because of the biases in their training and professional experience.

Results

While Dr. Wipke's group have been working on refining the operation of the INVENTON program, no new runs have been made with the YIGSR structure. The next runs will be made after incorporating our new data on the orientations of the required amino acid sidechains. This new structural input should provide a major refinement of the output "mimetics".

Discussion and Recommendations

We are very close to testing the first synthetic mimic designed by INVENTON (candidate 16 - Figure 7). The results of this will tell us whether we are close to our goal of providing a sufficiently active lead compound, or whether major modifications will be needed. These results will become available at about

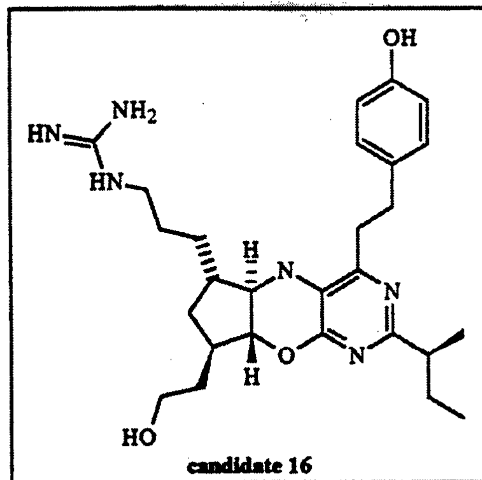


Figure 7. INVENTON candidate 16

the same time as the new runs from INVENTON, which incorporate more information on the required amino acid side chain orientations, are completed, and should greatly help their evaluation. Additional future runs with INVENTON will incorporate structural information from the binding pocket of the LBP. These will not be carried out for, at least a year due to the fact that we are only at the very beginning of the structural work on the ligand binging domain, C135 LBP.

Progress on Technical Objective 2, task 6.

Experimental Methods and Procedures

Dr. Konopelski has very nearly completed the synthesis of candidate 16 (Figure 7), with only minor changes from the protocol outlined in the original application. The synthesis was approached by making two fragments, each containing one of the outer core ring structures and appropriate sidechain mimics. The core central ring structure is formed when the two fragments come together in the final steps of the synthesis.

Assumptions

None

Results

The first potential mimic (candidate 16) will be tested for its bioactivity within the next few months. As indicated for tasks 4 and 5, the results of the tests will greatly facilitate the evaluation of the next INVENTON output.

Discussion and Recommendations

Results from the bioactivity tests of our first mimic designed by INVENTON, along with the new output from INVENTON which incorporates additional required sidechain information, will, in many ways guide the future direction of the design and synthesis side of this project.

Progress on Technical Objective 2, task 7.

This work will be initiated with the new set of runs on INVENTON which incorporate additional required side chain information.

Progress on Technical Objective 2, task 8.

This work will start with the receipt of candidate 16 by the Starkey lab.

Progress on Technical Objective 3, task 9.

Experimental Methods and Procedures

Anti-invasive activity of peptide 11 and its analogs is tested using an *in vitro* two-chamber "Transwell" assay. An 8 μm pore size polycarbonate filter separating the upper and lower chambers of

a 6.5 mm Transwell (Costar) is impregnated with a 1:20 dilution of Matrigel. 5×10^4 tumor cells are added to the upper well in 0.2 ml complete medium, and 0.8 ml complete medium was added to the lower well, and medium is changed daily. Incubation of the "Transwells" is continued for 3 days and cells invading into the bottom well are quantitated using the colored MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) metabolite. The lung colony assay is used to evaluate anti-metastatic activity. Tumor cells are harvested from subconfluent cultures with minimal trypsin/EDTA (Ethylenediaminetetra-acetic acid) exposure. Prior to injection, mice are warmed at 37°C for 30 minutes. A set number of monodispersed tumor cells are injected per mouse in 0.2 ml via the lateral tail vein. Where the experiment requires co-injection of tumor cells with peptide, cells are prepared as indicated above. One mg peptide dissolved in the injection buffer is first loaded in 0.1 ml into the syringe, then the aliquot of tumor cells is added in a further 0.1 ml. The contents of the syringe are gently mixed by inversion and injected as described above. The mice are killed several weeks later and autopsied. All tissues with suspect tumor colonies are rinsed in PBS and fixed in Bouin's fixative for gross and histological examination. The numbers of superficial nodules in the Bouin's-fixed tissues are determined using a dissecting microscope. While we have conducted most of our work to date with standard rodent test cell lines, we are now working more intensively with the highly invasive human breast cancer cell lines, MDA-MD-453 and MDA-MD-231. For these lines, *SCID* (Severe Combined Immunodeficient) mice are employed for the invasion assays, and spontaneous metastasis from the mammary fat pad is also assessed.

Assumptions

The general, and unavoidable, assumption made with the animal experiments is that human breast cancer cells will behave in *SCID* mice in the same way as they do in the human patient. There is also an assumption made that the "Transwell" *in vitro* invasion assay will reasonably predict anti-metastatic activity. After many years of experience with both assays, we are confident that the two assays give roughly parallel results for the type of work being done on this project. Certainly, the *in vitro* assay is a useful screen before going into animal experiments.

Results

The NMR experiments, carried out under our NIH award, to determine the active conformation of peptide 11 utilized Tr-NOESY (Transferred Nuclear Overhauser Effect Spectroscopy) experiments where the peptide interacted with purified LBP. Dithiothreitol is used to prevent dimerization of free peptide 11, but was not used in the presence of the LBP which has an internal disulfide bond. Detailed examination of Tr-NOESY spectra revealed that the bound conformation of peptide 11 was dominated by a structure which could not be distinguished from synthetic peptide 11 disulfide dimer. Since peptide 11 can spontaneously dimerize under the conditions used in these NMR experiments, the presence of peptide 11 dimer was initially viewed simply as an unwanted complication.

An analog of peptide 11 containing a para-fluoro substituted phenylalanine in place of the tyrosine residue was found to dimerize very much more slowly than peptide 11 in the absence of dithiothreitol (7-10 days compared with 6-7 hours for peptide 11 at room temperature at pH = 5.0). Surprisingly, this analog dimerized rapidly in the presence of the 67 kDa LBP (Figure 8). Rapid dimerization occurred when the peptide 11 analog was present in 32 fold excess of concentration over the LBP, and the rate of dimerization was found to increase with increasing concentration of the LBP. In this case, enzymatic activity of the 67 kDa LBP was suspected, and after appropriate controls were carried out, this was

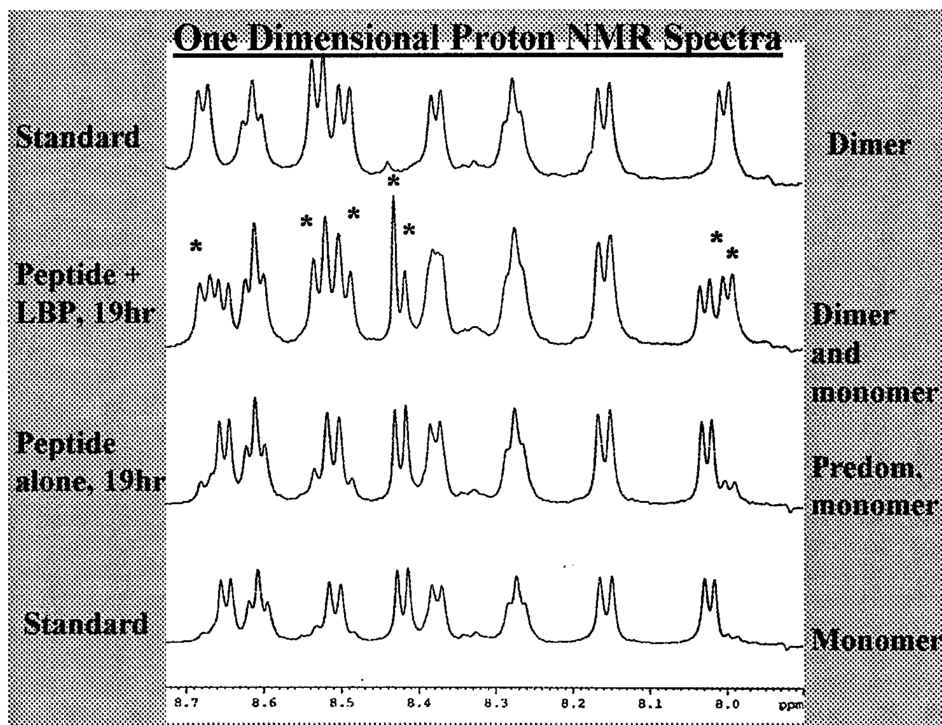


Figure 8. - One dimensional proton NMR spectra of the fluoro-phenyl alanine containing peptide 11 analog over time in the presence or absence of isolated 67 kDa LBP. After 40 hours in the presence of the LBP the peptide reached a 100% dimeric state with a spectrum identical to the top one shown in Figure 8. A 32 fold excess of peptide over LBP was used in these experiments.

confirmed. Thus, the 67 kDa LBP appears to have a protein disulfide isomerase activity. This activity was demonstrated for shed LBP isolated from conditioned tissue culture medium, for LBP isolated from EHS basement membrane matrix and for recombinant protein expressed in *E. Coli*. Given the hypothesis that the protein evolved as a laminin chaperone, and that it binds to an LE repeat region containing many disulfide bonded loops, this is a reasonable enzymatic activity (47), and one which could, by rearranging the laminin-1 disulfide bonds, locally alter basement membrane matrix conformation.

Our current theory is that such a local change in the matrix could facilitate tumor cell invasion. Preliminary experiments, quantitating invasion of DG44CHO cells through basement membrane matrix *in vitro*, revealed a significant increase of invasion over three days for matrix pretreated with 67 kDa LBP (Figure 9.). Given this preliminary data indicating that dimerization of peptide 11 might be involved in the bioactivities of the peptide, we compared the bioactivities of peptide 11, a non-oxidizable monomer of peptide 11 containing an Acm (Acetamidomethyl) protected cysteine residue and peptide 11 dimer. The disulfide dimer was slightly more active *in vitro* (anti-invasion) and *in vivo* (anti-metastatic) compared to peptide 11, but significantly more active than the Acm protected peptide 11 monomer (Table 2., Table 3.). The Acm protected monomer was the least active in each case.

Discussion and Recommendations

While any experimental data shedding light on the possible mechanisms of action of the 67 kDa LBP in facilitating tumor cell invasion and metastasis is very welcome, the apparent disulfide isomerase enzymatic activity of the protein does complicate the structural studies being carried out on the NIH award. Fortunately, the dimer is symmetrical, so all our previous work is valid. Since the minimal active sequence of peptide 11, YIGSR, is just as active as peptide 11 itself, YIGSR is still viewed as the most appropriate template for drug design. We expect to be able to elucidate why this short sequence is so active when we have determined the NMR structure of the LBP ligand binding domain. Most likely, the longer peptide requires interactions with the LBP at the N-terminal Cys residue as well as docking via the Tyr and Arg sidechains. The smaller YIGSR peptide may be able to fit into the binding pocket without the Cys interaction.

Progress on Technical Objective 3, task 10.

Work on this task will commence with the receipt of candidate 16 by the Starkey lab.

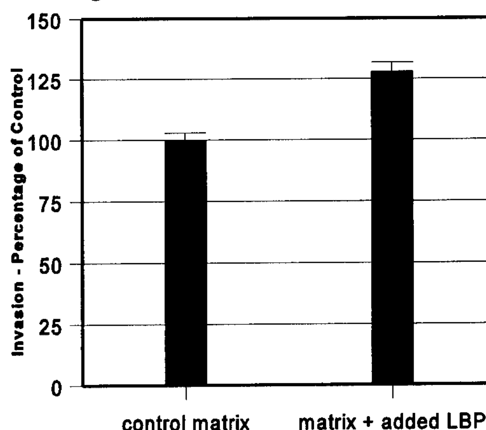


Figure 9. *In vitro* transwell assay for invasion. Filter separating upper and lower chambers impregnated with 1:20 dilution of matrigel or 1:20 dilution of matrigel exposed to 10% (by weight) isolated 67 kDa LBP for one hour before use. CHO cells were allowed to invade for 3 days, then the number of cells which had invaded were quantitated using MTT. Each data point represents 6 replicates.

Table 2. - Effect of Monomeric and Dimeric Peptide 11 on CHODG44 Cell Invasion of Matrigel Basement Membrane Assayed using the *In Vitro* Transwell Assay

Peptide[1]	% Inhibition of Invasion
Peptide 11	51.2 ± 3.6
Acm-peptide 11	11.4 ± 10.1
Peptide 11 dimer	75.7 ± 4.1

[1]100μg peptide per ml medium.
[2]Each data point represents 6 replicates.

Table 3. - Effect of Monomeric and Dimeric Peptide 11 on Experimental Metastasis Formation by B16BL6 Cells

Peptide[1]	% Inhibition of metastasis	p value[2]
None	None	-
Peptide 11	45	0.017
Acm-peptide 11	20	0.154
Peptide 11 dimer	51	0.007

[1]1mg peptide used per mouse.
[2]Mann-Whitney U 2-tailed test. [3]n=7 mice.

3

Conclusions

The major advances which have been made in the past year encompass the work aimed at elucidating the structure of the ligand binding domain of the 67 kDa LBP. Although difficulties with our isolation procedures for ligand derivatized LBP slowed down the studies on identifying the contact residues for peptide 11 in the ligand binding domain, the alternative approach of using phage display studies to pinpoint regions of interacting sequence worked very well. The phage display studies indicated that three separate sequence domains in the C-terminal domain of the LBP could interact with peptide 11. The phage display studies make a good start to mapping which LBP residues take part in peptide 11 binding. With new isolation procedures for ligand derivatized LBP in place, the fine mapping of actual contact residues is now also underway.

In order to undertake our NMR studies of the structure of the ligand binding domain of the LBP at reasonable cost, the binding domain needed to be expressed in a recombinant bacterial system. We have accomplished this both for the full length protein and for the ligand binding domain. In both cases, the *E. coli* strain expresses large amounts of poly-His tagged protein which is readily purified over a Ni affinity column. The poly-His tag is easily removed using thrombin cleavage, and the molecular weight of the expression products were confirmed by mass spectrometry. As judged by circular dichroism studies, the recombinant proteins refolded well. We are now at the point where we can start to carry out the initial NMR studies which will guide us as to the best conditions to use to collect the relevant spectra.

The synthesis of candidate 16, which was designed by one of the early runs of the artificially intelligent INVENTON program, is essentially complete, and will be provided to the Starkey lab for bioactivity testing very shortly. The results of these tests are very important, as they will indicate if the designed mimics are close to a potential drug lead, or if major modification to the INVENTON pharmacophore hypothesis will be needed. Refinement of the INVENTON input will be carried out within the next couple of months to incorporate new data on the orientation of required amino acid sidechains in peptide 11. This is key new data, crucial to effective drug design. Taken together with results of the activity tests for candidate 16, they should allow for substantial improvement of the INVENTON output. Additional refinement of the INVENTON input will largely come from the NMR studies on the structure of the ligand binding domain of the LBP. This additional refinement will not happen until quite late in the project because the relevant data will not be available till then.

Finally, because some of our initial experiments indicated that the 67 kDa LBP had a protein disulfide isomerase activity and could facilitate dimerization of peptide 11 analogs, we compared the anti-invasive and anti-metastatic activity of peptide 11, a non oxidizable Acm protected analog of peptide 11 and peptide 11 dimer. The most active species was the dimer, with the Acm protected monomer being the least active in each case. Interaction with the LBP via the N-terminal Cys residue is likely to be important to ligand binding of the longer peptide 11, while it appears not to be so for the short, very active, YIGSR peptide. Any structure which we derive for the "binding pocket" of the LBP will need to be compatible with these results. Along with requirements for disulfide isomerase activity, this consideration could provide some very useful constraints when we come to evaluate our future NMR derived structural data for the "binding pocket".

4

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