Heterologous expression of laminin peptide 11 on a virus particle surface for use in malignant tumor cell targeting
by Thomas Darmody Arnold

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University

Abstract:
This thesis details the construction of a chimeric tumor-targeted bio-imaging agent. There are two major components to this system: 1) the tumor-targeting ligand, which directs a host scaffold (macromolecule) to tumor tissues, and 2) the "host" scaffold, which carries imaging agents and displays, on its surface, the tumor-targeting ligand. Peptide 11 was chosen as the tumor-targeting ligand of the chimera. Peptide 11 (CDPGYIGSR), is a nine amino acid sequence derived from the β1 chain of the basement membrane molecule, laminin-1. This peptide is the major ligand binding domain for the 67 kDa laminin binding protein (LBP). The expression level of LBP shows a positive correlation with progression in many solid tumors. Synthetic peptide 11 blocks tumor lung colonization and tumor angiogenesis. Radio-labeled derivatives of peptide 11 have target and image tumor cells that overexpress LBP. We have chosen the protein capsid of Cowpea chlorotic mottle virus (CCMV) to serve as a macromolecular scaffold from which to present peptide 11. The CCMV scaffold has been chosen for its resilient and dynamic structural attributes and for its ability to bind large numbers of the magnetic resonance imaging contrast agent, gadolinium (Gd), on its surface. In theory, the CCMV-peptide 11 chimera will target multiple solvent exposed Gd ions to metastatic tumor cells expressing LBP on or near their surface. The specific aims of this thesis project are: 1) To design. an appropriate system for the construction of a protein engineered tumor-targeted bio-imaging agent; and 2) To genetically modify the coat protein subunit of CCMV to express exogenous peptide 11 at one of five possible exterior loop positions. The peptide 11 sequence has been inserted into four of the five surface loops of the viral coat protein and we have demonstrated stable virus assembly under various conditions. Systemic infection and efficient production of chimeras from plants appears to be compromised in some viral constructs. We are therefore using a novel yeast expression system to provide high concentration of empty capsid particles which can be loaded with contrast/therapeutic agents. Chimeras purified from yeast look and behave like native CCMV virions. The ability of these chimeras to target LBP, and eventually, to image metastatic tumor cells, will be investigated by others in this research group.

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APPROVAL

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Thomas Darmody Arnold

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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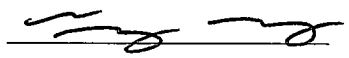
Signature 

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ABSTRACT

This thesis details the construction of a chimeric tumor-targeted bio-imaging agent. There are two major components to this system: 1) the tumor-targeting ligand, which directs a host scaffold (macromolecule) to tumor tissues, and 2) the "host" scaffold, which carries imaging agents and displays, on its surface, the tumor-targeting ligand. Peptide 11 was chosen as the tumor-targeting ligand of the chimera. Peptide 11 (CDPGYIGSR), is a nine amino acid sequence derived from the β1 chain of the basement membrane molecule, laminin-1. This peptide is the major ligand binding domain for the 67 kDa laminin binding protein (LBP). The expression level of LBP shows a positive correlation with progression in many solid tumors. Synthetic peptide 11 blocks tumor lung colonization and tumor angiogenesis. Radio-labeled derivatives of peptide 11 have target and image tumor cells that overexpress LBP. We have chosen the protein capsid of Cowpea chlorotic mottle virus (CCMV) to serve as a macromolecular scaffold from which to present peptide 11. The CCMV scaffold has been chosen for its resilient and dynamic structural attributes and for its ability to bind large numbers of the magnetic resonance imaging contrast agent, gadolinium (Gd), on its surface. In theory, the CCMV-peptide 11 chimera will target multiple solvent exposed Gd ions to metastatic tumor cells expressing LBP on or near their surface. The specific aims of this thesis project are: 1) *To design an appropriate system for the construction of a protein engineered tumor-targeted bio-imaging agent; and 2) To genetically modify the coat protein subunit of CCMV to express exogenous peptide 11 at one of five possible exterior loop positions.* The peptide 11 sequence has been inserted into four of the five surface loops of the viral coat protein and we have demonstrated stable virus assembly under various conditions. Systemic infection and efficient production of chimeras from plants appears to be compromised in some viral constructs. We are therefore using a novel yeast expression system to provide high concentration of empty capsid particles which can be loaded with contrast/therapeutic agents. Chimeras purified from yeast look and behave like native CCMV virions. The ability of these chimeras to target LBP, and eventually, to image metastatic tumor cells, will be investigated by others in this research group.

CHAPTER 1

INTRODUCTION

The ***overall goal*** of this thesis project is to begin development of a tumor-targeted bio-imaging system based on a plant virus scaffold (Cowpea chlorotic mottle virus - CCMV) – peptide 11 chimera. The CCMV capsid scaffold has been chosen for its resilient and dynamic structural attributes and for its unique ability to bind large numbers of gadolinium (Gd) ions (up to 180 ions per capsid). Peptide 11 was chosen as the tumor-targeting ligand of the chimera because it has been shown to specifically bind LBP shed from and/or on the surface of certain metastatic tumor cells. Although appropriate collaborations have already been arranged to evaluate the effectiveness of our constructs for bio-imaging applications, this thesis is limited to the their design, construction, and preliminary characterization.

The specific aims of this thesis project are:

1) *To design an appropriate system for the construction of a protein engineered tumor-targeted bio-imaging agent.* Structural elements (CCMV template scaffold and peptide 11) will be evaluated in a "rational approach" to designing chimeric CCMV-peptide11 capsid constructs.

2) *To genetically modify the coat protein subunit of Cowpea chlorotic mottle virus (CCMV) to express exogenous peptide 11 at one of five possible exterior loop positions.* Standard cloning techniques will be used to insert a peptide 11-based sequence into CCMV genomic cDNA. A variety of heterologous and homologous expression systems will be evaluated for their ability to generate the modified virion in a stable, assembled form. Assembled virions will be biochemically characterized.

CHAPTER 2

BACKGROUND AND PROJECT RATIONAL

Introduction

This thesis represents our *initial efforts* to design, engineer and test the potential for viral protein cages to serve as scaffolds for targeted delivery of cancer diagnostic agents. My thesis research details the construction of such an agent. The goal of this thesis background section is to introduce the two fundamental components that we used to create a protein scaffold-tumor ligand chimera and to briefly discuss why we chose these components. This is important since the goals of my thesis research (see Chapter 1, Introduction) originated from such a conceptual foundation. In the final section of this review, I will summarize the strategies that we used to create our tumor-targeted bio-imaging agent, and the criteria that we will use in evaluating its first forms. For a more in-depth look at "the cancer problem" that underlies my motivations for attempting this thesis, please see Appendix C.

A Two Component Chimera: Peptide 11 Plus CCMV

There are at least two fundamental components that make up a tumor-targeted macromolecular bio-imaging agent: 1) the tumor-targeting ligand, which directs the host scaffold to tumor tissues, and 2) a "host" scaffold, which carries imaging agents and displays, on its surface, the tumor-targeting ligand. We have chosen the protein capsid of Cowpea chlorotic mottle virus (CCMV) to serve as a macromolecular host and the LBP-seeking peptide 11, as its tumor targeting ligand. In theory, the CCMV capsid will target multiple solvent exposed Gd ions (MRI contrast agent) to metastatic tumor cells expressing LBP on or near their surface. Here, I will briefly review the two major components of our agent.

Why Peptide 11?:

Peptide 11 is the major ligand for the tumor cell surface receptor, laminin binding protein (LBP). In order to adequately discuss peptide 11 as a tumor-targeting ligand, its cognate receptor will first be discussed.

The 67-kDa Laminin Binding Protein (LBP). The 67 kDa LBP is a major nonintegrin cell surface receptor that mediates high-affinity interactions between cells and the basement membrane molecule, laminin-1. In 1983, two research groups independently reported the discovery of LBP, separated from extracellular matrix on laminin-Sepharose columns[1,2]. This protein was found to be expressed by a variety of mammalian cells and to be *particularly abundant on the surface of cancer cells*[3]. Several studies to elucidate this receptor's role in tumor progression have clearly demonstrated an increase in LBP expression in tumors compared with normal tissues[4,5], a correlation between LBP expression, invasive phenotype of the tumor[6,7], and poor clinical prognosis[8,9]. High expression of LBP is also associated with the invasive phenotype of normal embryonic trophoblastic tissues[10], migratory inflammatory cells[11], and embryonic cells during gastrulation[12]. The strong correlation between LBP overexpression and the invasiveness of certain cells in non-cancer situations and the metastatic potential of tumor cells suggests that this receptor plays an important role in the acquisition of a metastatic phenotype by cancer cells. (For a more thorough discussion on metastasis, please see Appendix C.) High expression of LBP is therefore a good biological marker of metastatic cells.

In an effort to characterize LBP, Rao *et al*, in 1989, isolated full-length cDNA clones from a human expression library using monoclonal antibodies against the purified 67 kDa protein[13]. Predicted molecular mass and *in vitro* translation of the selectively hybridized mRNA identified a protein of 32 kDa. Previous pulse-chase experiments had predicted this apparent LBP precursor protein[14], later termed laminin receptor precursor

(LRP). Posttranslational modification of this 37 kDa LRP including fatty acid acylation by palmitate, oleate and stearate may lead to dimerization of LRP, and provide a mechanism for the formation and plasma membrane association of functional LBP[15]. Interestingly, LRP is, by itself, associated with polysomes (and no cell surface localization) in oocytes and sea urchin embryos[16]. A homologous mouse protein, p40, is specifically associated with the 40S ribosomal subunit[17]. Moreover, duplicate protein homologues in yeast (YST1 and YST2) have been identified as essential protein components of the 40S ribosomal subunit. The assembly of these proteins into immature 40S subunits and the subsequent processing of 20S rRNA to 18s rRNA (through protein or catalytic RNA modifications) represent critical steps in defining the translational capacity of yeast cells[18]. Taken together, these results suggest a dual role for LRP in carcinogenesis: It may be involved in maintaining the translational capacity of cells (overexpression of the precursor protein in tumors could function to assure an adequate supply of active 40S subunits to meet the demands for protein synthesis during tumor cell growth and proliferation). As LRP dimerizes and is exported to the cell surface it then acts as a laminin binding protein (and extracellular matrix modulator). Recent phylogenetic analysis indicates that the laminin-binding function (specifically, the LMWWML palindrome from peptide G - a purported active site for laminin binding[19,20]) was acquired during evolution, around the same time that laminin-related molecules came to exist in nature[17].

Although the exact mechanism of LBP's action during tumor cell invasion and metastasis is not clear, recent data from the Starkey laboratory (Montana State

University, Bozeman, MT) may shed some light on this issue. In a recent study[21], chinese hamster ovary (CHO) cells were stably co-transfected with expression vectors for dihydrofolate reductase (dhfr), a full-length human β1 integrin chain, and a full-length α6 integrin chain. Growth in methotrexate (MTX)-containing medium resulted in amplification of both α6 and β1 expression products, which dimerized and then localized at the cell surface. The α6β1 integrin is a laminin-specific receptor that also appears to promote tumor cell migration, basement membrane matrix invasion, and metastasis[22] (and references therein). Upon MTX amplification and co-overexpression, subsequent surface expression of α6β1 integrin was increased. Endogenous LBP, under autonomous regulation in the cell (LBP expression appears to be regulated at the level of translation[22]), was concomitantly co-overexpressed with the laminin-specific integrin. Moreover, a significant amount of substrate-associated LBP (in addition to the LBP that was localized to the cell surface near focal adhesion plaques) was observed. This matrix-associated LBP may have been shed into the culture medium from tumor cells in response to the cells' interaction with matrix (or matrix components) via integrin mediated signaling. LBP shedding occurs in a variety of tumor cell lines and may be proportional to the invasive ability of the cells. It therefore seems likely that upregulation, and then induced shedding, of LBP promotes aggressive malignant behavior in solid tumors. The shed-LBP retains its ability to bind laminin[23] and is active in its ability to modify the basement membrane. Surface expressed and shed-LBP both appear to have protein sulfhydryl oxidase (SOX) catalytic activity[24]. Recent analysis demonstrating high similarity between the N-terminal region of LRP and various members of the chaperonin-

like HSP70 family lend support to the hypothesis that LBP could actively rearrange basement membrane laminin-1[19]. A possible model for LBP's role in invasion and metastasis might therefore include both tumor cell attachment via surface LBP-laminin interactions as well as an active reorganization of the basement membrane to facilitate cellular invasion.

The major ligand for LBP is the noncollagenous basement membrane component, laminin-1 (Fig 1). The laminin glycoprotein is composed of three chains, α_1 (400 kDa), β_1 (230 kDa), and γ_1 (220 kDa), arranged in a cruciform-like structure (as imaged by electron microscopy), with one long arm and three short arms each containing globular end regions [25,26]. The carbohydrate composition of laminin-1 is heterogeneous and specific sugar moieties are enriched on the globular end regions[23]. LBP exhibits a relatively high affinity for laminin-1 with a published K_d on the order of 2 nM[27].

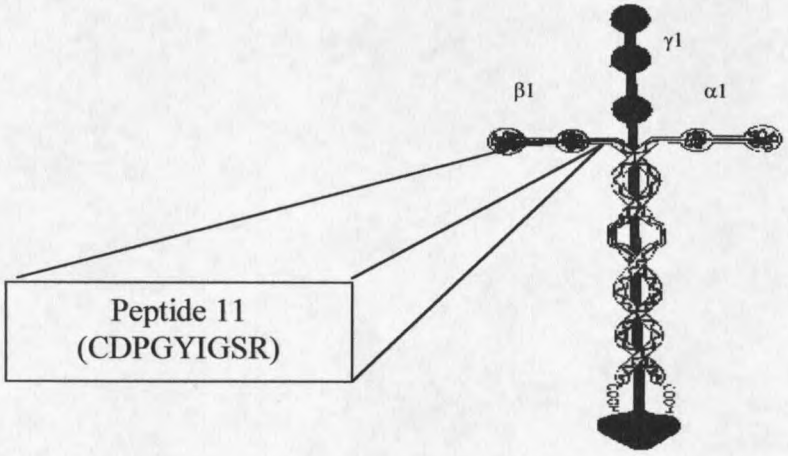

Fig 1. Cartoon depicting the basement membrane molecule, laminin-1 and the peptide 11 sequence found within the β1 chain.

Peptide 11. The nine amino acid sequence, CDPGYIGSR, known as peptide 11 (Fig. 1), is the primary ligand binding domain for the 67 kDa LBP. Peptide 11 is located within epidermal growth factor (EGF)-like repeats (LE repeats) in domain III of the laminin β_1 chain (residues 925-933)[23,26-28]. Free peptide 11, within purified protease-derived fragments of laminin or from direct synthesis of the small molecule, effectively blocks invasion of basement membranes by tumor cells[26,28,29], reduces experimental tumor lung colonization (metastasis)[28,29], and inhibits tumor angiogenesis[30]. Peptide 11 specifically elutes phage populations displaying mimotopes for three different regions of LBP (including the peptide G palindrome mentioned above) from a nine-mer random phage display library[20]. Also, a synthetic UV-light-activatable crosslinker based on the peptide 11 sequence (called the peptide 11 photoprobe) binds to and cross-links with surface and shed LBP[21]. This research demonstrates peptide 11's specificity for LBP, a molecule heavily expressed on the surface of tumor cells.

Alanine scan substitution experiments indicate that Tyr^5, Ile^6 and Arg^9 in peptide 11 contribute significantly to the anti-invasive activity of synthetic peptide 11. Based on structure:function experiments with D/L alanine for glycine analogues, free active peptide 11 tends to favor an "S"-shaped backbone conformation in solution[31]. Cyclic disulfide peptide11 (*-CDPGYIGSRC-*) is thought to possibly mimic the structure of the peptide 11 sequence found in laminin-1, since the peptide 11 sequence is derived from a disulfide bonded c-loop of an LE repeat. This cyclic isoform appears to adopt an S-shaped conformation[29] similar to the shape of non-cyclic isoforms, and exhibits good anti-invasive and anti-metastatic activity. Structure/function studies indicate that the