Albert B. Alkek Institute of Biosciences & Technology, Texas A&M

"The Structure and Function of Non-Collagenous Bone Proteins"
NASA-TMC Cooperative Agreement NCC 9-36

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Summary of Research

The research done under the cooperative research agreement for the project titled "The structure and function of non-collagenous bone proteins" represented the first phase of an ongoing program to define the structural and functional relationships of the principal non-collagenous proteins in bone. An ultimate goal of this research is to enable design and execution of useful pharmacological compounds that will have a beneficial effect in treatment of osteoporosis, both land-based and induced by long-duration space travel. The goals of the now complete first phase were as follows:

1. Establish and/or develop powerful recombinant protein expression systems;
2. Develop and refine isolation and purification of recombinant proteins;
3. Express wild-type non-collagenous bone proteins;
4. Express site-specific mutant proteins and domains of wild-type proteins to enhance likelihood of crystal formation for subsequent solution of structure.

The goals as stated in the initial proposal have been achieved, and are summarized in the following.

Expression Systems

Our laboratory has established and is effectively producing recombinant proteins using the following expression systems:-

a) prokaryotic systems pGEX, pMAL, and pQE; and

b) eukaryotic systems, baculovirus (insect cells), *pischia pastoris* (yeast), and vaccinia virus/T7 phage (mammalian cells).

These systems have been optimized for the production of high levels of recombinant protein.

Purification and Mass Production

Purification of recombinant proteins produced in all expression systems has been facilitated by use of fusion tags. Solubility of recombinant decorin produced in prokaryotic cells has been enhanced by fusion to a maltose binding protein; for expression of other proteins the poly-histidine sequence has proved to be most effective. The vaccinia expression system has been extensively refined by the development of five new expression vectors that will

facilitate both cloning, expression, and purification of recombinant proteins (vectors pT-cam1 through pT-cam5). Biglycan and decorin have been expressed in eukaryotic cells as fully processed glycoproteins and been extensively characterized (see *J. Biol. Chem.* 271, 19571 and *J. Biol. Chem.* 271, 19578). Mass production of proteins by prokaryotic (10-100 mg/l) and eukaryotic (20-40 mg/l) systems has now been achieved and preliminary crystallization studies initiated by our colleagues at UAB. Production of osteopontin in the prokaryotic system has yielded high levels of purified protein (see *Ann. NY Acad. Sci.* 760, 327), and crystallization is ongoing.

Mutant proteins

It is likely that the extensive post-translational processing non-collagenous bone proteins undergo may prove to be problematic to crystallization (although likely to be functionally important), therefore we are currently developing a range of mutant constructs that have deleted or modified carbohydrate attachment sites. In addition, we are now able to express recombinant proteins in mammalian CHO cells for which there exists a range of glycosylation mutants; biglycan and decorin constructs in which the glycosaminoglycan attachment site has been changed are being expressed in the Lec1 CHO mutant that will put a limited N-linked oligosaccharide on the core protein. Secondary structure appears not to be affected and mass production is underway for crystallization experiments.

Wild-type and truncated mutants of bone sialoprotein constructs have been developed for expression in eukaryotic cells; the recombinant protein produced by prokaryotes is insoluble due to the absence of correct processing.

Decorin and decorin truncates expressed in the pMAL system have been used to identify a divalent metal cation (Zn^{++}) binding site that may prove to be functionally important, in addition to possibly stabilizing structures for crystal formation.

In conclusion, progress on this project is extremely encouraging and we are hopeful of producing high quality crystals for X-ray diffraction in the current phase of the program.

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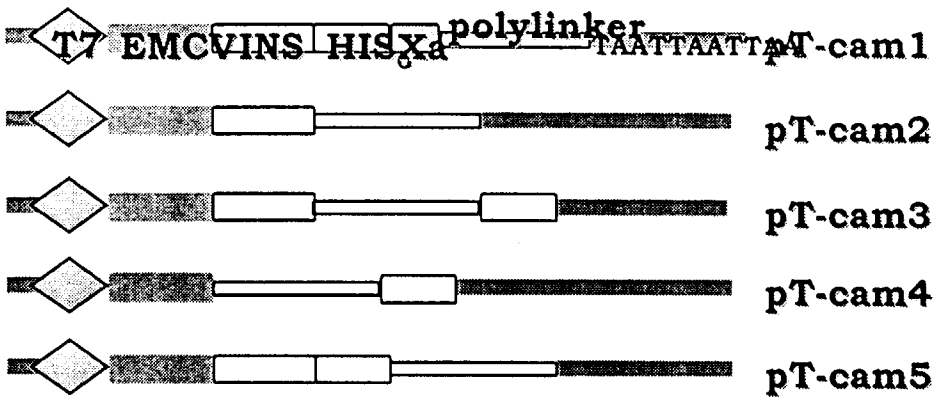
PROGRESS REPORT (Part of Final Report, Round I)

The versatility of the vaccinia expression system has been greatly expanded by the development of a panel of fusion protein expression constructs (Fig. 1). These plasmids, pT-cam1 through pT-cam5, enable expression of matrix glycoproteins in eukaryotic cells with or without an exogenous insulin signal sequence (to target nascent protein for processing and secretion), a poly-histidine domain either at the N-terminal or C-terminal end (for rapid, single-step affinity purification), and contain an extensive multicloning site for convenient insertion of heterologous genes.

Biglycan and Decorin, small interstitial proteoglycans that can regulate collagen fibrillogenesis *in vitro* and appear to be important in modulating the action of TGF-β (an agonist of osteoclast and osteoblast activity) have been expressed in large quantity (>20 mg per experiment) by use of recombinant vaccinia viridae. Biglycan has been expressed both as a proteoglycan (i.e. with 2 GAG chains) and as a core protein form (i.e. devoid of GAG chains) containing two N-linked oligosaccharides. Crystal formation is more likely with the later form so culture conditions were modified so that chemical amounts of purified core protein have been purified and are currently undergoing crystallization screening at UAB. Decorin similarly has been expressed as both a proteoglycan and core protein form; however, the core protein is variably substituted with 2-3 *N*-linked oligosaccharides. We have generated site-specific mutations in the core protein so that both the GAG attachment and the 3rd oligosaccharide attachment site have been deleted; expression of chemical amounts of homogeneous core protein is underway and will be sent for crystallization. Both biglycan and decorin possess extensive secondary structure (far UV CD-spectra), and this project represents the first time that these molecules have been produced in significant amount with maintenance of secondary structure. The likelihood of finding conditions for crystal formation and subsequent solving of the structure is high.

Recombinant vaccinia viridae encoding full length bone sialoprotein (BSP) and epiphycan as poly-histidine fusion proteins have been generated and are now ready for large scale production. BSP is a bone-specific protein and undergoes osteoblast-specific post-translational modifications; expression will be done using osteoblast cultures propagated in the STLV system of Dr. Neal Pellis (NASA). Similarly, epiphycan expression is restricted to the growth plate of developing cartilage and it therefore likely that cultures of growth plate chondrocytes that maintain an appropriate phenotype in the STLV system will be optimized for large scale production.

(Fig. 1)


T7
EMCVIN
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Xa
polylinker
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