GRANT NUMBER DAMD17-96-1-6211

TITLE: Integrin Regulation of Apoptosis in Breast Cancer

PRINCIPAL INVESTIGATOR: Dana Hu, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, CA 92037

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Using homology PCR and RACE, I cloned a novel gene from breast carcinoma cell line MCF-7. This ADAM protein, which is designated as ADAMx, has the highest homology to the monkey fertilin I. The protein sequence reveals that it constitutes the following domains characteristic of the ADAM proteins: a prodomain that contains the putative cysteine-switch cysteine residue, an active metalloprotease domain as evidenced by the existence of the zinc-binding motif, a disintegrin domain comprising highly conserved cysteine arrangement pattern found in all soluble snake venom disintegrins and previously identified ADAMs, a cysteine-rich domain that contains a fusion peptide, a well defined EGF domain and a putative transmembrane domain. Most importantly, ADAMx is not found in highly and moderately metastatic breast carcinoma cell lines MDA-MB-435 and MDA-MB-231. Experiments will be focused on the interaction between the disintegrin domain and integrin receptors. The ultimate goal is to define the role of ADAMx in tumor metastasis and apoptosis.
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 7/31/97
Date
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRONT COVER</td>
<td>1</td>
</tr>
<tr>
<td>REPORT DOCUMENTATION PAGE</td>
<td>2</td>
</tr>
<tr>
<td>FOREWORD</td>
<td>3</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>4</td>
</tr>
<tr>
<td>BACKGROUND AND SIGNIFICANCE</td>
<td>5</td>
</tr>
<tr>
<td>BODY OF REPORT</td>
<td>7</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL METHODS AND RESULTS</strong></td>
<td>7-12</td>
</tr>
<tr>
<td><strong>DISCUSSION/CONCLUSIONS</strong></td>
<td>12-13</td>
</tr>
<tr>
<td><strong>FUTURE PLANS</strong></td>
<td>13</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>14-16</td>
</tr>
</tbody>
</table>
A. Background and Significance

Uncontrolled cell-extracellular matrix (ECM) interactions result in matrix turnover and tumor invasion. The cell-ECM interactions are coordinated series of events composed of modification of cell-cell binding and cell-ECM adhesion, proteolytic degradation of ECM and cell de-attachment, migration through ECM and entering into the circulation. The integrin cell surface adhesion bind to matrix proteins play important role in these processes. When unchecked, these interactions result in over-proliferation, circumvention of apoptosis and thus tumor growth and metastasis [1,2 and 27,28]. Matrix metalloproteases (MMPs) and membrane-type metalloproteases (MT-MMPs) also constitute one of the pivotal points of ECM turnover [3-6]. MMPs and MT-MMPs are intimated involved in the malignant phenotype as shown by numerous studies including those using the human breast carcinomas. This current study focuses on a newly cloned membrane protein, ADAMx, which contains both a metalloprotease domain and an integrin binding domain. The identification of this protease protein in the breast carcinoma cells suggests that ADAMx may play a role in breast tumor metastasis and invasion and the likely mechanism of action is through ADAMx - integrin interaction and the metalloprotease activities.

The ADAMs are a family of complex membrane proteins which mostly resembles the MT-MMPs (Figure 1). ADAMs are usually composed of a pro-protein domain (PP), a metalloprotease domain (MP), a disintegrin-like domain (DIS), a cysteine-rich domain (CR), a transmembrane domain (TM) and a cytoplasmic tail (CT). Some of the ADAMs also have EGF-like motifs (EGF) and fusion peptides (FP). To date, about 20 members of the ADAM family proteins are cloned from a variety of species and tissues [7-9].

The ADAMs' PP and MP domains have the characteristics of MMPs and MT-MMPs, that is, most MP domains contain the consensus zinc-binding sequence HEXXH and many ADAMs also contain a potential "cysteine-switch" cysteine residue in the PP domain. Thus, like the MMPs and MT-MMPs, the protease activity in ADAMs may also be controlled by the "cysteine switch" mechanism [10,11].

![Figure 1. Domain arrangement of several types of metalloproteases](image-url)
The DIS domain of the ADAMs share striking structural homology to the snake venom peptide disintegrins, as demonstrated by their highly conserved locations of the cysteine residues. The snake venom disintegrins, which contain the RGD/KGD "integrin recognition sequence", are ligands for integrins [12,13]. Thus, the ADAMs are suggested to be integrin ligands. Unlike the peptide disintegrins, though, most ADAM disintegrins lack the RGD/KGD sequence in the presumed integrin-binding hairpin loop. In fact, the corresponding residues in the ADAM disintegrins are quite degenerate. One explanation is that the structure and the position of the loop, not the exact amino acid sequence, is key for integrin binding [14]. This is supported by the fact that mouse fertilin-β, an ADAM protein, binds to integrin α6β1 using the TDE sequence within the disintegrin domain [15].

What are the biological functions of the ADAMs? To date, most data about the ADAMs are generated from cell fusion studies. It is reported that ADAMs fertilin-α and β participate in sperm-egg fusion [7,15,16]. It is reported that during the sperm-egg fusion, the fertilin-β binds to integrin α6β1, resulting in the exposure of the fusion peptide in the fertilin α-subunit which, in turn, allows the fusion. ADAMs are also implicated in cell differentiation. For example, mouse meltrin-α was shown to play important role in myoblast differentiation and fusion, leading to myotube formation [7,17]. The latter is an important process in skeletal muscle generation. Another interesting function of the ADAMs is proteolysis of degradation and remodeling of surface proteins and ECM components, as the closely related snake venom metalloproteases (SVMP) do [7]. One study showed that TACE (tumor necrosis factor-α converting enzyme), an ADAM protein, can induce the release of TNF-α from tumor cells, presumably by cleaving membrane-bound TNF-α precursor [18]. Another ADAM, ADAM-11, is a candidate human cancer tumor suppressor, as its gene is disrupted in both the breast and ovarian cancers by somatic rearrangements [19]. In conclusion, the ADAM family proteins are likely involved in interactions with ECM components and with cell surface proteins, including the integrins. Therefore they most likely play functional role in tumorigenesis.

I have cloned a novel ADAM protein (ADAMx) from non-invasive human breast epithelial cell line MCF-7. ADAMx is not found in the MDA-MB-231 or MDA-MB-435 human breast carcinomas which have mesenchymal (fibroblast-like) morphology and are highly invasive [20,21]. This expression pattern is opposite to that of the MMPs and MT-MMPs, both of which are found to be expressed in the invasive cells, but not in MCF-7 cells [22]. The difference in the expression patterns leads to the hypothesis that ADAMx, as part of the ECM remodeling machinery, may act through a pathway different from that of the MMPs and MT-MMPs. It also suggests that ADAMx may be important for maintaining breast cell epithelial morphology and that the loss of ADAMx could link to tumor progression and metastasis.
B. Experimental Methods and Results

B.1. Identification of ADAMx From Breast Cancer Cell Lines

To study the correlation between ADAM gene expression and tumor invasiveness, I have initiated experiments to identify ADAMs in several human breast cancer cell lines, including MDA-MB-435, MDA-MB-231 and MCF-7. The 435 cells represent advanced tumors because they are highly invasive, the 231 cells are moderately invasive and the MCF-7 cells are not invasive; Thus the latter cell line represents early stage tumors [20,21]

The initial cloning was performed by homology RT-PCR method using total RNA from the three cell lines, oligo dT<sub>20</sub> and a pair of degenerate primers derived from conserved sequences within ADAMs' disintegrin domains. The sequence of the primers are: sense (DIS-1): 5'-RSD-GAR-SAG-TGT-GAY-TGT-GG-3' and antisense (DIS-2): 5'-GCA-AWW- TTC-WGG-RAR-RTC-RCA-3'. Both sequences were derived from the respective conserved peptide sequence GEECDCG and CDLPE(L/H)C within the disintegrin domain of known ADAMs. The primers were synthesized by Integrated DNA Technologies Inc.. The reverse transcription reactions were carried out as follows: total RNA sample (1-5 µg) from the cells was incubated with oligo dT<sub>20</sub> (100-500 ng) and an aliquot of H<sub>2</sub>O at 65°C for 10 minutes, then at room temperature for 2 minutes and cooled down on ice. A final reaction volume of 20 µl containing this mixture, RNase Inhibitor, dNTPs, sodium pyrophosphate, reverse transcription buffer (10x) and 0.5 unit of AMV reverse transcriptase (Promega) was put in a thermal cycler and the reverse transcription (RT) reaction was carried out at 45°C for one hour. The reaction tube was heated at 95°C for 2 minutes and quickly chilled on ice. Another 0.5 unit of reverse transcriptase was added and RT reaction repeated once more. To amplify the cDNAs, 1µl of above RT product was mixed with the degenerate primers (100-200 ng each), dNTPs, 10xPCR buffer, H<sub>2</sub>O and one unit of Taq polymerase to a final volume of 100 µl. Polymerase Chain Reaction (PCR) was carried out for 32 cycles (95°C, 30sec; 48°C, 1min; 72°C, 1.5min). A final extension step was performed at 72°C for 15 minutes. All materials were purchased from Boehringer Mannheim unless otherwise noted. Figure 2 below shows a result of a RT-PCR.

Figure 2, lane 1, 1Kb DNA ladder; lane 2, 435 cell RNA; lane 3, 231 cell RNA and lane 4, MCF-7 cell RNA. Each sample lane contains one fifth of the PCR reaction volume. The expected size of the PCR product is ~180bp which is marked with the arrow. This is a representative of three RT-PCR experiment repeats.
Sequence analysis revealed that the MCF-7 cell RNA contains one novel ADAM protein. I designated this novel ADAM as ADAMx. In addition, human homologue meltrin-α and metargidin was also found in MCF-7 cells. Several ADAMx gene-specific primers were synthesized according to the sequence information derived from these experiments. RT-PCR using the gene-specific primers for ADAMx confirmed that there was no detectable ADAMx messenger in the invasive 231 and 435 cells (not shown). ADAMx is also identified in a human osteosarcoma cell line (MG63, not shown).

B.2. Cloning Full Length ADAMx Gene

I used two approaches to clone the full length ADAMx gene. One was the Rapid Amplification of cDNA ends (RACE) [23,24] and the second is to screen the human cDNA libraries. This was done by both me on an human placental cDNA library (Invitrogen) using the filter-lifting method (see below) and by a commercial service (Research Genetics, Huntsville, AL) on the human BCA library using high density membrane hybridization method.

B.2a. RACE experiments

RACE (Rapid Amplification of cDNA Ends) was used in order to clone the rest of the sequence of ADAMx gene. To perform 3'-RACE, first, reverse transcription reactions were carried out using oligo dT20 or oligo dT17-Ro where Ro is an adapter primer. Then, cDNA was amplified using an upstream ADAMx gene-specific primer and dT20VN or Ro. Often, nested PCR was necessary to further amplify gene specific products. In this case, another pair of primers inside the first pair were used and PCR was carried out as usual. To perform 5'-RACE, the reverse transcription reactions were carried out using a downstream ADAMx gene-specific primer. The cDNA was purified from the reaction mixture (to remove the first primer, RNA and enzymes). First the cDNA was treated with NaOH to denature all the enzymes and then neutralized by adding HCl. The cDNA was then purified by using the QIAquick Spin PCR Purification Kit (QIAGen) according to manufacturer’s instruction or using the Microcon-30 concentrators (Microcon). The cDNA was then tailed with poly(A) nucleotide using dATP and terminal transferase (Promega) at 37°C for half an hour. The transferase was denatured by heating the reaction mixture at 65°C for 2 minutes. cDNA was then purified by Phenol/Chloroform extraction and ethanol precipitation. This pellet was brought up in TE buffer and was used in PCR experiments. Finally, double stranded DNA was synthesized using oligo dT17-Ro and a downstream gene-specific primer (gs-1). Subsequent nested PCR was performed using Ro and another gene-specific primer inside the first gs-1.

As a result of the RACE experiments, at least 80% of the ADAMx gene was cloned (this is based on the sequence comparison to other known ADAM genes). An alignment of the putative integrin-binding loop of several ADAM's disintegrin domains is shown in figure 3. Note that in ADAMx, RGD sequence that appears in metargidin (ADAM-15) is replace by a SRS sequence.
Figure 3. Sequence alignment of "integrin binding loop" among several ADAMs. Here MG63/MCF7 represents ADAMx gene.

The confirmed protein sequence of ADAMx is shown in figure 4 (below).

...LCQHPALWKNQVALEEAKIKFQTWAPQKWNLRLGLVPGPSCIRLEILMLLVIFVPSMYCHLGSIYYSFYEIIIPKRTLTVQGGDSPVEGLSYLLLMQGQKHLVHLKVKNRFVNNFPVYSYNHGGLLQQESPFISHDCHYEYIEGMSGSFVSVNICAGLRGTSSLRKRKNLTALSPWTLDQGLNMCTPMHIKRESPVVSTSWQQGRKPHDLQALSYCJHSHKKYVEMFVWHNNRFQMWGSNVNETQTVVDIALANSFTRGINTEVLAGMEIWTEDGIDLDTVLDQITLRNFHWRQEMFFHRAKHDVAHMVGHHPGQNMQAFLSGACSSGFAAAVFHFHEDVLLFAALMAHELGHNLGQHDHSCAFCKDKHFCLMHENITKESGFSSCDDYFYQFLREHKGACLFNKPRPRSRKRRDSACGANGVVEDTEQDCGSLCQHHACCDECILKAKAESCGPCCHKCKFHRKGYPCPSSRSCDLPEFCNGTSALCPNNRHQDGSKCHTIYECLKVHCMDPNNQCLQLYGYGAKSASQEYNSMNSKGDFQGNCISTSGPSQYVRCSDNSGIFCGKLICSGITGLPKINLQHTMIQPQGDGCWSDMAYSTDIPDEGDVHNTYCAPNKNVCLNSACTDKTPVISACNPKKTCNGKVCNLGHCHCEGHAPDCVTAGSGGSVDGLPGKLGGPSPGEHENHMHSSRREEHAVDMMLILSFIILFILLSTII*SACLKNHQRPLPRQKFLQQWHLHRPQK*SQKQQWPQKIKKKKHMWPT

Figure 4. The sequence of ADAMx (incomplete). The zinc-binding motif in the metalloprotease domain is double underlined. The disintegrin domain is in bold. The potential fusion peptide is underlined and the putative transmembrane region is in italics. The 3' coding region, 5' coding region and untranslated region of the gene remain to be completed.

The functional motifs/domains of ADAMx were identified by comparison of the sequence to those of known ADAMs. Sequence homology search using the NCBI BLAST Search showed that ADAMx has the highest protein sequence homology with
the fertilin molecules (ADAM-1) from mouse, guinea pig and macaque [25]. In some regions, ADAMx's amino acid sequence is 50-70% identical to the fertilin molecules. An alignment of partial sequence of ADAMx with fertilin alpha molecules is shown (see attachment). Therefore, ADAMx seems to be a human homologue of fertilin alpha.

The above sequence information revealed that ADAMx contains a pro-domain, a metalloprotease domain represented by the presence of the conserved Zn-binding peptide sequence HELGHNLGIQH; The MP domain is followed by a disintegrin domain, a cysteine-rich domain, an EGF-like domain and a transmembrane domain (Figure 5). The TM region is predicted by hydropathy plot analysis (Figure 6).

![Figure 5. Domain arrangement of ADAMx](image)

![Figure 6. Kyte Doolittle hydropathy plot of ADAMx (partial sequence). The potential fusion peptide is marked by an arrow and the predicted transmembrane region is blackened.](image)
B.2b. Library screening

Double stranded DNAs of ADAMx encoding either the disintegrin domain of ADAMx or the disintegrin domain plus 5' end of the cysteine-rich domain were amplified by PCR and gel-purified twice. These samples were used to synthesize the \([\alpha]^{32}P\)-radio-labeled DNA probes using the Random Primed DNA Labeling Kit (Boehringer Mannheim) according to manufacturer's recommendation. The human placental cDNA library in E.coli (MC1061/P3) was titered and the appropriate amount of the bacteria culture was plated on LB-Amp agarose. Overnight colonies were transferred to membrane filters (Millipore, 0.45µM) by filter-lifting. The colonies on the filters were washed, lysed, fixed and then cross-linked to the membrane by an UV Crosslinker. The filters were allowed to hybridize with either of the two labeled DNA probes at 68°C overnight in the "QuikHyb" solution (Stratagene). The filters were then washed by buffers of low stringency and then high stringency. The dried filters were exposed to the X-ray film (Kodak) for autography. At least 18 positive clones were picked and the corresponding bacterial culture grown from which second round library screening were conducted. After this, however, no positive clones were found.

Meanwhile, the same \([\alpha]^{32}P\)-radio-labeled DNA probes described above were sent to a commercial company (Research Genetics, Hunstville, AL). The human BCA cDNA libraries were screened by high-density membrane hybridization. After several attempts, they were able to identify four I.M.A.G.E. clones that were positively hybridized to my DNA probes. However, PCR amplification of these clones using ADAMx-specific primers yielded negative results. Therefore, these clones must be non-specific hybrids. The above results strongly suggest that ADAMx is a rare gene. Next, I plan to screen a genomic DNA library and/or screening MCF-7 cDNA library in order to obtain complete sequence of ADAMx.

B.3. Polyclonal antisera production

I have raised polyclonal antibodies against the disintegrin domain of ADAMx. To make the antigen, the DNA of DIS domain was PCR amplified with primers each containing a restriction enzyme cleavage site. The PCR product was cloned into expression vector pET15b (Novagen) using restriction sites Xho I and BamH I. The protein was expressed in E. coli bacterial BL21 (DE3) cells upon IPTG induction. The recombinant protein was found to be retained in the inclusion bodies and thus had to be solubilized by using 6M urea. The protein contains a N-terminal His-tag and was coupled to a Ni\(^{2+}\)-affinity Sepharose 6B column (Figure 7). The protein was eluted off using 1M imidazole in 6 M urea/PBS. The sample was dialyzed against 6 M urea/PBS to remove imidazole.
The purified and concentrated antigen was used to immunize rabbits. Briefly, 150μg antigen was mixed with Complete Freund's Adjuvant and was injected into each New Zealand white rabbit. Pre-bleed sera were collected prior to the injection. The rabbits were immunized six more times with 100μg of antigen every two weeks.

The ability of the antisera to recognize recombinant disintegrin domain of ADAMx was confirmed by ELISA. The disintegrin domain of ADAM-15 [26] was used to examine cross reactivity (Figure 8).

[C. Discussion]

This project focuses on the study of a novel gene, ADAMx, found in human breast tumor MCF-7 cells. ADAMx's partial protein sequence reveals that it constitutes the following domains characteristic of the ADAM proteins: a prodomain, a metalloprotease domain that contains the zinc-binding motif, a disintegrin domain comprising highly conserved cysteine arrangement pattern found in all soluble snake venom disintegrins and previously identified ADAMs, a cysteine-rich domain, a fusion
peptide, a well defined EGF domain and a putative transmembrane domain. So far, experiments have focused on DNA cloning and antibody production.

It is known that soluble metalloproteases (MMPs) and membrane-type metalloproteases (MT-MMPs) are involved in basement membrane degradation, cell growth regulation and tumor invasion. The snake venom peptide disintegrins are ligands of cell surface receptor integrins, a family of proteins intimately involved in ECM turnover and tumorigenesis. Based on the fact that ADAMx a disintegrin domain, my hypotheses is that, by interfering the interaction between cell surface integrin and ECM proteins, the ADAMx's disintegrin domain may alter adhesion and migration properties of tumor cells.

Interestingly, ADAMx is found in the non-invasive MCF-7 cells that retain epithelial morphology whereas it is not expressed MDA-MB-231 and MDA-MB-435 breast carcinoma cells, which have lost epithelial phenotype and are highly invasive. This expression pattern is opposite to that of MMPs and MT- MMPs, both of which are highly expressed in the two invasive breast carcinoma cell lines. This differential expression patterns suggests that ADAMx may be important for maintaining epithelial morphology. This is indicative that ADAMx may be important in controlling cell growth, proliferation.

Together, ADAMx may be a key determinant in the invasive and metastatic potential of breast epithelial carcinoma.

D. Future Plans

I will devote my effort in the following studies to generate information about ADAMx.

1) complete the cloning and sequencing of ADAMx. This includes the confirmation of the 3' and 5' coding regions of the protein.

2) express individual domains of ADAMx, especially the active disintegrin domain in the baculovirus/insect cell system. This will provide powerful reagent for the study its binding to integrins.

3) assess cell type and tissue specificity of ADAMx gene and gene product by Northern and Western blotting.

4) study the role of ADAMx in cell adhesion and migration by using the ADAMx-transfected MDA-MB-435 cells.
E. References


metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 85, no. 5:683-693.

# MAP Multiple Sequence Alignment Results

## Page 1.1

<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>15</td>
<td>16</td>
<td>30</td>
<td>31</td>
<td>45</td>
<td>46</td>
<td>60</td>
<td>61</td>
<td>75</td>
</tr>
<tr>
<td>2 GP</td>
<td>MRSGLAMASVRNTS FSAALQKHARVLHNAR</td>
<td>SRLQCQTLMMVAPRL</td>
<td>LGVPEHCLRVRLTVK</td>
<td>LLGVTLQPHIHHCL</td>
<td>GPVHYSSYEVIPES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>4 Monk</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>5 rabbit</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>

## Page 2.1

<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>91</td>
<td>105</td>
<td>106</td>
<td>120</td>
<td>121</td>
<td>135</td>
<td>136</td>
<td>150</td>
<td>151</td>
</tr>
<tr>
<td>2 GP</td>
<td>LTVGSSQDQPRGRTSY MLQIQQHQLVLHKLK</td>
<td>KRDYVDDFPYVYSH</td>
<td>NGNVRQETPSIARDC</td>
<td>HYEGYIEGASSSFVS VSACGSLRGI--LIK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>4 Monk</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>5 rabbit</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>

## Page 3.1

<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>181</td>
<td>195</td>
<td>196</td>
<td>210</td>
<td>211</td>
<td>225</td>
<td>226</td>
<td>240</td>
<td>241</td>
</tr>
<tr>
<td>2 GP</td>
<td>ENTSYIEPILOLSQR FEHLYMMAQAPVS</td>
<td>CRASSQDSQAVTSW</td>
<td>QGSRKPSQVQALSS</td>
<td>YLMVHTKVEMFVVO NNQLIPOMGWGSDNET</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>4 Monk</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>5 rabbit</td>
<td>GETSYSIEPILOLSKR FEHALYTMHGAHVS</td>
<td>CSVTKKQGQGMSTSR</td>
<td>QQGSELKLNQALSS-</td>
<td>YLWSHTKVEMFVMN DNQRFQOMGWMGRVSET</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## Page 4.1

<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>271</td>
<td>285</td>
<td>286</td>
<td>300</td>
<td>301</td>
<td>315</td>
<td>316</td>
<td>330</td>
<td>331</td>
</tr>
<tr>
<td>2 GP</td>
<td>VQAVMDIALANSFT GINTEVVLGQLEIW</td>
<td>TEGDPFVPVDLQQT</td>
<td>LNFNPWFRQKLVGR</td>
<td>VRHDVAMELGHRP</td>
<td>ENEQQLRGAASCSE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>4 Monk</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>

http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl

7/15/97
### BCM MAP Multiple Sequence Alignment Results

#### Page 2 of 5

**5 rabbit** VQRVMDIIALANSFT RGINTEVVLGMEIW TEGDLTEVAAD1QVT LRFNFSWRQEQVLVR VRTDVAHMIVGRHPG ENITGQALFGACCGG

---

### Page 5.1

<table>
<thead>
<tr>
<th></th>
<th>361</th>
<th>375</th>
<th>376</th>
<th>390</th>
<th>391</th>
<th>405</th>
<th>406</th>
<th>420</th>
<th>421</th>
<th>435</th>
<th>436</th>
<th>450</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>FAFAVEAFHHEVDLL</td>
<td>FAALMAHELGHNLGI</td>
<td>QHDHTCTCGPKHFC</td>
<td>LMGEKIGKDSGSFSC</td>
<td>SSDHFLRFLHHRGA</td>
<td>CLDLDEPGRSRMRRA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 GP</td>
<td>FAFAVEAFHHEDLA</td>
<td>SAILVHELGHNLGI</td>
<td>RHDHSAFCVCDKHC</td>
<td>LMENITKEGSFSC</td>
<td>SSDYEHFLHHRGA</td>
<td>CLFNPWHRARRR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>FAFAVESFHHEVDLL</td>
<td>FAALMYHELGHNLGI</td>
<td>QHDSACFCEKHC</td>
<td>LMHETKESGFSNCE</td>
<td>SSDYPFQFLREHKG</td>
<td>CLFNPKRPRSRKRRD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Monk</td>
<td>FAFAVESFHHEDEL</td>
<td>FAAMVFHELGHNLGI</td>
<td>QHDSACPCRKHFC</td>
<td>LMHENITKESGFSNC</td>
<td>SSDYPFQFLREHKG</td>
<td>CLFNPKPRPRSRKRRD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

### Page 6.1

<table>
<thead>
<tr>
<th></th>
<th>451</th>
<th>465</th>
<th>466</th>
<th>480</th>
<th>481</th>
<th>495</th>
<th>496</th>
<th>510</th>
<th>511</th>
<th>525</th>
<th>526</th>
<th>540</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>ACGNGVVEDLEEC</td>
<td>CGSDCSDFCCSCPTC</td>
<td>TLKEAQCSEQLCCY</td>
<td>NCTFSSKGLRCPAE</td>
<td>DVCFLPEYCDGTSQ</td>
<td>CPANSIMQDGTDRC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 GP</td>
<td>ATCGNGVVEESSEQ</td>
<td>CGVNCDCSDECDQAC</td>
<td>LNKGNACSNELSSC</td>
<td>DCYKNSGYLRCFSV</td>
<td>GPCDFLPEYCTQGSK</td>
<td>CPLDTYKDGTPCNE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>SACGNGVVEDEQ</td>
<td>CGSLQHHACDENC</td>
<td>ILKAKAESCDPGCS</td>
<td>KCKHFHKYPCPS</td>
<td>RSCDLPEFCNGTSAL</td>
<td>CPNNRHQDKDGSKCHT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Monk</td>
<td>SACGNGVVEDEED</td>
<td>CGSACHLDFCSSCPTC</td>
<td>TLKEAECQSHCLGC</td>
<td>DCTFRKGRKFLRCPQ</td>
<td>DELCFLPDESSGA</td>
<td>CPADSYKQDGTPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

### Page 7.1

<table>
<thead>
<tr>
<th></th>
<th>541</th>
<th>555</th>
<th>556</th>
<th>570</th>
<th>571</th>
<th>585</th>
<th>586</th>
<th>600</th>
<th>601</th>
<th>615</th>
<th>616</th>
<th>630</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>IYYCLGGWCKNDKQ</td>
<td>CRYGYPARASPE</td>
<td>CYSVINTPKRNGFC</td>
<td>GHFTSNAFRYTEDS</td>
<td>EDVFCGLKVTDDVY</td>
<td>LPKVPLHSLIQVY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 GP</td>
<td>GEFCVSGWTQPC</td>
<td>CATYFHCGRAPAP</td>
<td>CTLYDNSIGNIFGC</td>
<td>GQSGNP-TTVCSCS</td>
<td>DSKCGLKICTGSS</td>
<td>IPPAIRLAFAIQIP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>IYECVNHMDFDNQ</td>
<td>CLQVLGYGAQASQ</td>
<td>CYNMSMKGDPQFC</td>
<td>GSTPSPGYQYRCS</td>
<td>GNIFCGKLCSTT</td>
<td>LPKINLQHTMIQVPQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Monk</td>
<td>IYHCSCGGQCPDKQ</td>
<td>CNYSIYGRAPASED</td>
<td>CYSMNTRGDRFGNC</td>
<td>GHTPETOQYTVTCS</td>
<td>DNVFCGLKICTGQSVS</td>
<td>LPRVKAQHTVQIPH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

### Page 8.1

<table>
<thead>
<tr>
<th></th>
<th>631</th>
<th>645</th>
<th>646</th>
<th>660</th>
<th>661</th>
<th>675</th>
<th>676</th>
<th>690</th>
<th>691</th>
<th>705</th>
<th>720</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mouse</td>
<td>GEDWCSMDAYN-T</td>
<td>DVPDDGVQSGSFC</td>
<td>PNVKCMEDYCTRGTV</td>
<td>LQYNECFQPXMCHGNG</td>
<td>VCNFINCKHCCHDAGFA</td>
<td>PPDCSSPGNGSVD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 GP</td>
<td>GDDWCWSINFGDPA</td>
<td>STSPTEGASAVSGTSC</td>
<td>SKGACVNACSFSTTL</td>
<td>DTANCSSAEACMACNE</td>
<td>ICNLHGHCGDGFPGA</td>
<td>PNNCEQOTGGSVS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Adam-X</td>
<td>GDGSCSMMYSN-T</td>
<td>DIPDEGQHVGYTCA</td>
<td>PNVCVLSNACTDTSPT</td>
<td>VISACNFKTPCTNGK</td>
<td>VCNLGHCHCNECHA</td>
<td>PPDC-TAGSSSVS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Monk</td>
<td>DNDWCWSMADN-T</td>
<td>DTPDNGHVGTSCA</td>
<td>PKVNTCDYSCVHBS</td>
<td>LLYDRPFEESGCNGH</td>
<td>VCNLRLHCHCNGGFS</td>
<td>PPDCNQPGNGSVD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

5 rabbit **EDDWCWSIDSN---S** GCSDYGDVQRTYCA LNVKCDHSCVYQA PNSDCQADEMGKKGV CYNFRHCHDGSYGA PPDCRNPCTGGSVD

---

http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl

7/15/97
BCM MAP Multiple Sequence Alignment Results

Page 9.1

1 mouse 721 735 736 750 751 765 766 780 781 795 796 810
2 GP 0.000000
3 Adam-X
4 Monk

5 rabbit GPGKPYRNN-ISSS TN-S-SRTIKKSE NLNVLFFVPIFLII VLCVLLISYLWSEV KSVVSSIAESKEESE ESSEELPSEESVEAP

Page 10.1

1 mouse 811 825 826 840 841 855 856 870 871 885 886 900
2 GP
3 Adam-X
4 Monk

5 rabbit PPEQPAQQEAPQQ EA-PAIREAPP--E AARPAEAP-PPPE-- QAPP-EQA PPEAEPKPAEAPPP EAAPPQAPPPPEEA

Page 11.1

1 mouse 901 915 916 930 931 945 946 960 961 975 976 990
2 GP
3 Adam-X
4 Monk

5 rabbit PPEAPPPEEAAPP EAPPPEEAAPP EAPPPEAPP EAPPPEEAAPP EAPPPEAPP 919

Alignment Data (Fasta format)

>mouse

http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl
BCM MAP Multiple Sequence Alignment Results

GPVGPADFRHLSLPLAEESPPDDKMEDEEVNLKVMVLFVIFLYVLLCMLIAYLWSEV
QEVVSPSSESSSSSSSSSDSSQ----------
-----------------------------

>GP
MRSGSMMSAVRTNISFSALQKHVVHLVHARSILQCTTCLLMKVAPRLGLYPGLHLCVRLTVK
LVVGTLLPHHICHLGFVHYSSYEIVIPESTLVGSKQDPRGRTSYMLIQQKLQHILHV
KRDFVDDDPFVYSYHNGNVRQPETSIPARDCHYEGYIEGASSSVFVSACGLRGI--LII
ENTSYGIEPISSLQRFVHVLTYMQAPVQVSRASAKDSQVRSTSWQGQSKPPHVSQAGSS
LYVWLYCTVEFVVMQVRMFGWSTGQERQVTQVRVDVIALANIFTGRINTEVLAVGQVEW
TEGDLLIEIPVDRVLRLRFNURNWRQDKLLPRVHDVAMIVHHPGETSSQAFNLGACSSG
FAAVEAHFHDALASSALMLVHELGHNLGIRHDSACVRDKHSLCMQENITESSFNSC
SSDYFHYFLHEHRGACLNFKHAKRRRAARATCGNGVEESEQDCGVNCDTSCCCDDQC
NLKGNCSNCELCSDCQYKNSGFCRPSGCPDCIPETQGQSKCPDLYDKQDGGFCE
GFCYSKOGCTDIQCATYFGHGASAPACYTTLNSIGNIFGNCQSGNP-ITYVGCSSG
DSTKCGKLCTGISSIPPIRALFAAIQIFFHDDCWISINFGDPAHPEGAHAVSGTSSCA
SGKACVQUQSTFTLDTANCSSAAEMCNENIGCNNLGHCDDGFAPPNCQEQTGGSDS
GPPP---SS-TPTAPPKTQTQKASSENALIGIIILVLLLLVICAICL----------
-----------GI
FAEAPPTEPEEEAGEELEEEPEPEPEEEAEED----------
-----------------------------

>Adam-X
-----------------------------
LCQHPALWKNQVALEEAKIKQTFWAPQKWNRLGLVGPSCIRLEIL
MLL-VIFVPSMYCHLGSIYFYYFIIIKPRLTVQDDPSGLELYLIMQOCKHVIHLHV
KRHHFVNNFVYSHYNGLLGQFFISHDCHFEGYEIGMSVSFVNPICAGLRTSSLK
EEKSYSPERMDDSSRFHEVLYTHMACQ--------
-----------------------------
SFTGRINTEVVLAGNEIW
TEGDLLIDTVLQITLRFNWNHRQEMFHRKHAKHDVAMIVHHPQNSMQAGLSGACSAG
FAAVERSFHHEDVILFAAMAHREDLHNGLIQHDHSACPKDHFKHFCLMENITKESFGCSS
SSDYFQPILEHHKGCALNFKPRPSRRKRDSACSGNVEQTEDQGCSLQOHHCDDENC
ILKAAKACSDGPCCHKCFFRHGYPCCPSRSSCCLKPEFCNGTSLACPNNKHODGSKCTK
IYECVLKVCMDPNNQCLQLYGYGAKSASQPCEYNSMNSKQDFQNGCISTSPGSYVRCSD
GNIFCNGKLCSTQITGLPKINQLQHTMIQVFOQGDGSCWMDAYMS-TDIPEDGHDNFGTYCA
PNKCVLNSACTDKTPFVISAACNPPTCNGKVCGNCDLGHCNEHGHAPDC-TAGSSGSSVD
GLPGKLGTP-SEGEEHNNTHSREEHAVDMILSTFI-LFILLSTISS----------

ACLKHNQRLPRQKFLQWHLHMRPKQSKQKQKWPKKHKMFPW----
-----------------------------

>Monk
-----------------------------
---MSVIALLKDSANILLILWKSQVALEEVKKIKQTFWAPQKWNLRLGMLVGPSCIRLEIL
LIL-VIFVPSMHICLGLSIYFYYEIIPKRLTVQDDPSGLELYLIMQOCKHVIHLHV
KRSHFNVMFVYSYHNGILQGESPFTSHDCHYEIGYIEGVSFS-SVNTCAGLRTG--LII
ENTSYGIEPISSLQRFVHVLTYMQAPVQVSRASAKDSQVRSTSWQGQSKPPHVSQAGSS
LYVWLYCTVEFVVMQVRMFGWSTGQERQVTQVRVDVIALANIFTGRINTEVLAVGQVEW
TEGDLLIEIPVDRVLRLRFNURNWRQDKLLPRVHDVAMIVHHPGETSSQAFNLGACSSG
FAAVEAHFHDALASSALMLVHELGHNLGIRHDSACVRDKHSLCMQENITESSFNSC
SSDYFHYFLHEHRGACLNFKHAKRRRAARATCGNGVEESEQDCGVNCDTSCCCDDQC
NLKGNCSNCELCSDCQYKNSGFCRPSGCPDCIPETQGQSKCPDLYDKQDGGFCE
GFCYSKOGCTDIQCATYFGHGASAPACYTTLNSIGNIFGNCQSGNP-ITYVGCSSG
DSTKCGKLCTGISSIPPIRALFAAIQIFFHDDCWISINFGDPAHPEGAHAVSGTSSC

http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl
BCM MAP Multiple Sequence Alignment Results

TLKEHAECSHGLCCLDCTFRKGFCLRPQDECDLPEYCDGSSAECPAPSYKQDGTLCDR
IHVCSSGQCKNPNDQVNIYGPAPRSAPICYISMNTGDFGCNPHTEDQTYVTCSD
DNVFCKEILCTGQSLPRVKQHTVIQVPHDNDCWSMDADI-TDTPDNNGNIVGTSCA
PKVCTDYSVCVHSSLILYDCRPEESCHGKGVCNNLHRCHCQSGPPAQPCDKNPNGGSVD
GPPGQVTNN-SESGSE-DECARGQLRDQVYKLVVLFLVVLLLCSLLTISYLCSEV
QTAVAEVESSTETTLESE-LTSDLV-----------PIAEILPPGEEAPPPEE
APQPGPEETLPPGE--------EAPQPGEPETLPPGEAPPAEAAPPPEA-----PPPEAA
PPPEAPPEAPPEAPAPEAAAPPQAPPP--EAPPPEAPPQAP-

> rabbit
---MLATTARSVSSSLLSYFQPMINGAAARPQSWPVQMNGLRLGLVPFSLRVLGT
LLWGMIFLSIYMEI- VHYSSYEMVIEPLITVGESEKPEEASYLIFMQQKQLVHLH
HKDDFVVDFVYSHYNRLGQEMFLISRNCEYEGYIDGVPSFVSVTCSQGLGV---LVK
GETSYSIEPLSKSRFEHALYTMAGAHAVSCVTSKGGQGMSTRQGSRKLNQPALS3
YLWSHTKSVEMFVVNQRFQVMWVNETVQVMDIAALANSFTRGINTEVIVLAMEIW
TEGDLTVEADLQVLTRFNSWRQEOQQLVRVHRHDVAMIVGRHPGENTQQAFLNGAFIGSS
FAAAXESFHHEDILLFAALMAHELGHNLGIQDHSACTCNQPFFCLMGEINTKESSFSCN
SSDDYRFPLREHGACLFINPKRHSRTRLISRCGNGVETPEQCDEPDALKDCPCDSSMC
RLKDNQCGYGLCCFRCKYRRGFICRSRIGNCDLEYSCGKASCPDPAYKDQGTFPCR
VRCLQGGQCMNPDKQCSNYIGIPARSAPECYVILMSKDRFGNCSPAPPQSSLYVQGAD
ENIFGKLICTEVKLPQIQHQTVIQTAYEDGWCSIDSNN---SGCSDYQVQRTNYCA
1NKVCKDSHCYVQAPNSDQADEMCSQKGV CNNRFHCHDSGYAPPDCRNPGTGSSVDS
GPPPQKYBNR- ISSSTN-S-SRITKKKSENENLYFVFVPIFLLIVLCVLILWLYE
KSVVVSIESKESESSEELPSSEVESAPPEEQPAQQQAFAQQAFAAAPPAPPEAPPPEAPPEAPP

---Kim C. Worley, Human Genome Center, Baylor College of Medicine
kworley@bcm.tmc.edu

http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl

7/15/97