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Ching Yuan Hu

In recent years, the general public has recognized the dangers of a high fat diet and are demanding meat with lower fat content. This demand has stimulated research in the growth and regulation of adipocytes. However, despite much effort, no adipocyte-specific plasma membrane markers from any species are available as an aid to accurately distinguish adipocytes from non-adipocytes. One potential candidate for such a marker in porcine adipocytes has been identified by Killefer and Hu (1990b). Characterization of the cDNA for this protein, designated porcine adipocyte membrane protein (PAMP), is presented here. Sequence for the 910 bp clone is 80% similar to an internal region of a rat prostaglandin $F_{2\alpha}$ receptor regulator protein (FPRP) described by Orlickey (1996). Western blot analysis suggests that the pig protein is a homotetramer held together with disulfide bonds which form very close to the transmembrane region making the tetramer extremely difficult to reduce to monomeric units. Oligonucleotide primers were designed to amplify a genomic fragment by the polymerase chain reaction (PCR) and for a reverse transcriptase PCR (RT-PCR) assay to study the expression of the mRNA. A 2114 bp genomic clone revealed one intron in the coding region. A serum-free primary cell culture system was used to study the expression of the mRNA. Although message was detected every day over a ten day period, it appeared to peak between 6 to 8 days after plating. The PAMP protein is clearly of the same family as the rat FPRP but its size and conformation are quite different so it is not clear what function it performs in porcine adipocytes. Further experiments should focus on attaining full length cDNA's, confirming the molecular conformation of the protein, and assessing its function in a serum-free primary cell culture system.

Characterization of a cDNA Encoding a Porcine Adipocyte Membrane Protein

by

Kevin L. Vergin

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Redacted for Privacy

Major Professor, representing Animal Science

Redacted for Privacy

Chair of Department of Animal Science

Redacted for Privacy

Dean of Graduate School

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Kevin L. Vergin, Author

ACKNOWLEDGEMENT

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INTRODUCTION

Adipose tissue in animals is an important storage site for high energy triglycerides. Fat tissue is deposited in several different areas of the body. These depots vary in structure and function and may be separately regulated. While this organ is necessary for life, excess fat storage can have detrimental health effects. In animal production, leaner animals are desired both by consumers because meats with less visible fat are judged to be more healthy (Rathje and Ho, 1987) and by producers for more efficient feed conversion.

Despite intense efforts, relatively little is known about adipocyte growth and development. One major problem is the difficulty of distinguishing pre-adipocytes from other cells. While it is easy to distinguish an adipocyte with a monolocular fat droplet, it is nearly impossible to identify the same cell when it is devoid of fat. Thus, precursors to mature fat cells are very much like other cells and thus, factors affecting their differentiation and regulation are unknown.

Pre-adipocyte studies would be greatly enhanced if a pre-adipocyte-specific plasma membrane protein was identified which could serve as a marker to distinguish adipocytes and pre-adipocytes from other cells. Killefer and Hu (1990b) have identified a possible candidate marker by generating a monoclonal antibody against porcine adipocyte plasma membrane. Previous studies of the protein's expression have demonstrated that the protein is present in adipocytes but not in nine other tissues. In addition, the protein appears to be expressed in phenotypically lean pigs but not obese pigs (Killefer and Hu, 1990a). This result implies that the protein may be involved in adipocyte regulation such that the lack of the protein may lead to obesity. This study attempts to further characterize this potential marker by analyzing its nucleotide sequence and deduced amino acid

sequence. The expression of the mRNA for this protein was also studied by developing a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The characterization of this protein could yield important clues for studying pre-adipocyte growth and development.

LITERATURE REVIEW

In recent years, the general public has been educated about the dangers of high fat diets. As is typical with affluent cultures, people tend to indulge in foods containing higher fats including choice cuts of meat (Rathje and Ho, 1987). These diet choices can lead to obesity which has been associated with a variety of health problems. Through education campaigns, peoples dietary choices can change. In a study in Arizona, it was discovered that in 1983 all groups studied showed a significant increase in the amount of visible fat trimmed from cuts of meat. Unfortunately, probably due to a lack of knowledge, consumption of ground meats and processed meats containing high amounts of fat increased (Rathje and Ho, 1987). It would seem that people are aware of the dangers of a high fat diet but are not always knowledgeable of all the sources of fat. Some people would prefer to avoid all meat but this is a drastic choice which removes a valuable source of nutrients from the diet. Meats are an excellent source for the B vitamins, iron, zinc, and essential amino acids and, in addition, have more digestible proteins and a greater bioavailability of nutrients than any non-animal source (MacDonald, 1991). If meats are to have an important role in the diets of most people, then fat content of these meats should be reduced to avoid long term health problems.

One reason that change in the fat content of meat is slow to occur is that the USDA, which inspects and grades meat, rewards cuts with higher fat content. Meat producers have therefore traditionally raised animals to maximize fat content (Bergen and Merkel, 1991). One method to reduce fat content in growing animals is through the use of chemicals and hormones such as steroids, growth hormone, and ß-adrenergic agonists. Steroids tend to decrease fat content while growth hormone tends to increase muscle accretion. ß-adrenergic receptors are stimulated naturally by epinephrine and norepinephrine which stimulate lipolysis leading to decreased adipose stores. The ß-adrenergic agonists are synthesized compounds which imitate the actions of the natural

ligands. These methods are labor intensive and the effects of these compounds on other tissues and reproductive capacity are not fully understood. Spurlock, et al. (1994) show that pig adipocytes become desensitized to the agonists so that the lipolytic effect is diminished with prolonged exposure to the compounds. Also, while the ß-adrenergic receptors are well characterized in the rat, it is not clear which receptors are present on pig adipocytes (Mersmann, 1996). A better method to reduce adipose tissue in animals would be a site-directed method where adipocytes would be specifically targeted without affecting other tissues. The first step in this method is to identify proteins which are unique to adipocytes. Membrane bound proteins would be ideal targets since circulating compounds would be able to recognize these targets without first entering the cells.

While a few adipocyte membrane proteins have been studied, these have not proven to be adipocyte specific (Abumrad et al., 1993; Zhou et al., 1992; Kang and Chiang, 1986). Recently, however, Killefer and Hu (1990b) have identified a candidate protein from porcine adipocytes which, on the basis of western blots of several tissues, appears to be tissue specific. Therefore, this candidate protein will be referred to as PAMP (porcine adipocyte membrane protein). Attempts to further characterize this protein are described here. Sequencing of the PAMP cDNA has revealed a relationship to a previously discovered protein from rat reproductive tissue (Orlickey and Nordeen, 1996). This protein's postulated function is a membrane bound regulator of a receptor for prostaglandin $F_{2\alpha}$ (Orlickey, 1996). As will be seen in the results of this study, the nearly complete porcine protein sequence is clearly of the same family as the rat FPRP. However, it is not identical and, therefore, the function of this protein is not clearly demonstrated. Intriguingly, there are studies which show clear relationships between prostaglandins and adipocyte growth and regulation (Ramwell, 1973). Indeed, prostaglandin $F_{2\alpha}$ has been shown to be produced by adipocyte cell lines and primary cultures (Gao and Serrero, 1990). Also, recent studies by Kliewer, et al. (1995) and Forman, et al. (1995) demonstrate that a derivative of prostaglandin J_2, 15d-PGJ_2, can interact with peroxisome

proliferator-activated receptor γ (PPARγ) to induce adipocyte differentiation. PPARγ is a nuclear receptor which forms a heterodimer with retinoic acid receptor (RXR) and then acts as a transcription activator for several proteins involved in adipogenesis. However, there is no evidence to support a connection between prostaglandins and the PAMP protein.

The PAMP protein is thought to be a multimeric protein consisting of four identical subunits. Even this multimeric form is not as large as the rat FPRP. It is further hypothesized that FPRP and PAMP are alternatively spliced versions of the same gene. Alternative splicing mechanisms have been reported in the same tissues (Parruti et al., 1993) and cell lines (Juvonen and Pihlajaniemi, 1992) as well as tissue and cell line specific forms (Matsuda et al., 1993). In at least one case, alternative splicing of the 5' end of the pp52 mRNA lead to the loss of a potential Ca^{2+} binding site which could have affected the ultimate function of this variant (Gimble et al., 1993).

While significant progress has been made with the PAMP protein, much is still unknown. Conclusive evidence confirming the cloning of the entire mRNA is a top priority. Attempts to ascertain the function of the protein should be aided by its relationship to the rat FPRP. In addition, this protein's expression can be studied in a serum-free cell culture system.

MATERIALS AND METHODS

cDNA Library Construction and Sequencing

A cDNA library was constructed from porcine adipose mRNA as described by Killefer (1992). Briefly, total RNA was isolated according to the method of Chomczynski and Sacchi (1987). An oligo-dT cellulose column was used to isolate mRNA which was then used to construct a cDNA library using the Lambda Zap II cloning vector (Short, 1988) as described by the manufacturer (Stratagene, Inc., La Jolla, CA). The recombinant phage library was screened by inducing expression of the ß-galactosidase fusion protein and plaques expressing the fusion protein of interest were detected immunologically using a monoclonal antibody, LA-1 (Killefer and Hu, 1990b) in combination with an alkaline phosphatase reaction. Positive plaques were cored, titered, and rescreened two additional times to ensure clonality. Positive phages were then converted to pBluescript phagemid vectors as described by the manufacturer (Stratagene, Inc.). *E. coli* strain XL1-Blue hosts transformed with the plasmid vector were rescreened with the monoclonal antibody LA-1. A clone, 52B, was selected for further analysis.

Clone 52B plasmid was amplified and purified using the alkaline lysis protocol (Birnboim and Daly, 1979). Plasmid was bidirectionally sequenced manually using an ^{35}S-labelled dideoxy terminator protocol according to the manufacturer's instructions. Sequence was confirmed bidirectionally using an ABI 373A automated sequencer and dye terminator chemistry. Sequence was analyzed using the Genetics Computer Group (GCG) software developed at the University of Wisconsin (1987).

Electrophoresis and Immunoblotting

Clone 52B, containing the cDNA for PAMP, was induced to produce fusion proteins as described by Maniatis (Sambrook et al., 1989) Briefly, *E. coli* cells containing 52B plasmid were grown overnight at 37° C in Luria Bertoli (LB) media containing 25 µg/ml ampicillin. A 1 ml aliquot of culture was removed and then subcultured in 4 mls of fresh LB-ampicillin at 37° C. After 1 hour, IPTG was added to the culture and cells were further incubated at 37° C for 4 hours. Two mls of culture were then pelleted in a microcentrifuge at 15 K rpm. Supernatant was removed and cells were resuspended in loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) containing no reducing agents.

Porcine adipocyte plasma membrane proteins were prepared from dorsal subcutaneous adipose tissue removed from 50-100 Kg contemporary (typical market) pig phenotypes during slaughter. Adipocytes were isolated from adipose tissue by collagenase digestion (Rodbell, 1964) and a self forming Percoll gradient was used to separate plasma membranes (Belsham et al., 1980). Porcine adipocyte plasma membrane proteins were resuspended in gel loading buffer as above. Dithiothreitol (DTT) was made fresh by resuspending in 50 mM MOPS to a concentration of 1 M. For reduced samples, DTT was added to protein samples resuspended in gel loading buffer to make a final DTT concentration of 400-500 mM.

Samples containing 15 to 20 µg of total protein were incubated for 1 hour at 37° C, heated to 95° C for 2 minutes , put on ice and loaded onto a 12% or 15% SDS-PAGE gel (Laemmli, 1971). Colored molecular weight markers from either Sigma (Kaleidoscope; St. Louis, MO) or Amersham (Rainbow; Arlington Heights, Il) were used. Electrophoretic separation was achieved using 200 V for 45 minutes on a BioRad sub-cell apparatus (BioRad, Hercules, CA). Proteins were then transferred electrophoretically to Hybond ECL nitrocellulose (Amersham) using Tris-Glycine-Methanol buffer and a transfer apparatus from Idea Scientific. Proteins were transferred overnight at 150 or 250 mA.

Proteins were detected using an Enhanced Chemilumenescent (ECL) Western Blotting Detection Kit (Amersham) with minor modifications. Briefly, after transfer of proteins, nitrocellulose was treated with 5% non-fat dry milk in PBS-T (140 mM NaCl, 17 mM K^+, 10 mM PO_4^-, pH 7.4, 0.1% Tween-20) for 1-2 hours at 4° C to block available protein binding sites. The nitrocellulose was then incubated with a fresh dilution of LA-1 primary antibody (50 μg/ml)(Killefer and Hu, 1990b) in PBS-T for 30-60 minutes at room temperature with shaking. Blots were washed with PBS-T and then incubated with goat anti-mouse IgM antibody conjugated with horseradish peroxidase for 30-60 minutes at room temperature with shaking. Both antibody dilutions were determined empirically. Blots were washed again with PBS-T and then soaked in detection reagents for 1 minute, wrapped in Saran Wrap, and exposed to X-ray film for five hours or overnight. Film was developed using an X-omat automatic processor and then analyzed by densitometer (Molecular Dynamics, Sunnyvale, CA). Data were processed using FragmeNT Analysis software from Molecular Dynamics.

RT-PCR

Experiments to optimize conditions for the reverse-transcriptase polymerase chain reaction (RT-PCR) assay are detailed in Appendix A. Pre-adipocytes were isolated from 1 day old pigs and cultured as described previously (Akanbi, et. al, 1994). Total RNA was isolated from cell culture plates essentially by the method of Chomczynski and Sacchi (1987) except that cultures were first rinsed with PBS and then lysed with 4M guanidinium thiocyanate. Plates were scraped and the resulting lysate was transferred to centrifuge tubes. The rest of the isolation was unmodified. RNA was stored in 70% ethanol until assayed. RNA was then dried and resuspended in ddH_2O. For the RT-PCR assay, 500 to 1600 ng of total RNA was added to the reverse transcription reaction which

consisted of 10 mM Tris-Cl, pH 8.3, 90 mM KCl, 200 uM dNTP, 1 uM reverse primer (5'

ATC TCC GCG CTC AGA ACT TG), 2.5 mM $MnCl_2$, 5% acetamide, and 0.75 U Tth

DNA polymerase and incubated as follows: 94°C for 5 sec., 55° C for 30 sec., 65° C for

10 min. for 2 cycles. After first strand synthesis, 2 ul of the RT reaction was added to 48

ul of a PCR reaction consisting of 10 mM Tris-Cl, pH 9.0, 100 mM KCl, 750 uM EGTA,

0.05% Tween-20, 5% glycerol, 200 uM dNTP, 200 nM each primer (forward - 5' CTC

CTG TCA TCC CTG GAT CG), 1.5 mM $MgCl_2$, 5% acetamide, 1.9 U Taq DNA poly-

merase and incubated as follows: 94° C for 20 sec., 55° C for 30 sec., 72° C for 2 min. for

65 cycles. All PCR assays included a negative control which used water in place of the

RT reaction and a positive control which used less than one ng of PAMP cDNA plasmid.

PCR products were analyzed by electrophoresis in a 1% Seakem LE gel (FMC Corp.,

Rockland, ME) and documented with an Ultra-Lum gel documentation system (Ultra-

Lum, Inc., Carson, CA).

Dorsal subcutaneous fat samples from genetically lean and obese pigs (Scott et al.,

1981) were collected as described for cDNA library construction.

Ten tissue samples were collected from each of three crossbred market weight

female pigs during slaughter. RNA was isolated from dorsal subcutaneous adipose, brain,

heart, kidney, muscle, liver, large intestine, small intestine, stomach, and spleen using the

method described previously.

Southern Blotting

Porcine genomic DNA was isolated from liver using standard methods (Sambrook,

et. al, 1989). Approximately 50 Units of *Bam*HI or *Hind*III restriction enzyme was used

to digest twenty to thirty micrograms of porcine genomic DNA. Digested genomic DNA

was electrophoresed through a 0.8% agarose gel and blotted onto nylon membrane by capillary transfer essentially according to Sambrook, et. al (1989). A *Hind*III digest of lambda DNA was used as a molecular weight standard and 5 ng of the 910 bp PAMP cDNA was used as a positive control. The PAMP cDNA insert was random labeled using a commercial kit and ^{32}P-α-dATP according to manufacturer's instructions (Amersham). Probe was hybridized to the blot overnight at 65° C and washed at a stringency temperature of 65° C essentially according to Sambrook, et. al (1989). The blot was exposed to a phosphorimager plate and detected with a Molecular Dynamics Phosphorimager. Data were processed using FragmeNT Analysis software from Molecular Dynamics.

Intron Sequence

Genomic DNA from porcine liver was amplified by PCR using the same reaction conditions as for the RT-PCR assay. This product was purified and ligated into a pCRII vector (Invitrogen) according to manufacturer's instructions. Transformed clones were isolated and plasmid was purified using a Qiagen Qiaprep Spin Plasmid Purification Kit and sequenced as described previously except samples were analyzed using an ABI 377 automated DNA sequencer.

Database Submission

Sequence for the 52B (PAMP) clone and the genomic intron have been deposited in Genbank.

RESULTS

A cDNA library was constructed for adipocyte mRNA and fusion proteins from plaques were screened using a monoclonal antibody (LA-1) which was determined by F-ELISA to recognize an adipocyte plasma membrane protein (Killefer and Hu, 1990b); one clone was chosen for further analysis. Restriction digestion of the plasmid with Xho I and Xba I yielded an insert of 910 nucleotides. The sequence was determined bi-directionally using both automated and manual sequencing methods. The longest open reading frame, designated porcine adipocyte membrane protein (PAMP), is indicated below the nucleotide sequence in Figure 1. This sequence was then compared to all sequences in the GenBank/EMBL databases and one sequence, the rat prostaglandin $F_{2\alpha}$ receptor regulator protein (FPRP) with an 80% similarity in nucleotide sequence was found (Orlickey and Nordeen, 1996). The FPRP gene cDNA is 5782 nucleotides which includes 2067 nucleotides 5′ and 2847 nucleotides 3′ to the region of similarity to PAMP. The region of overlap includes the last 192 amino acids of the FPRP open reading frame plus 295 nucleotides of 3′ untranslated region. The predicted amino acids for each open reading frame are 91% similar with 71% of the amino acid changes being conservative changes. Interestingly, there are two regions of 49 and 84 nucleotides each with 33% similarity to the FPRP gene in the 3′ untranslated region. One region is immediately after the stop codon and the other is just before the poly A tail of PAMP. The rest of the 3′ untranslated region is 90% similar which is very close to the level of conservation of the open reading frame.

```
PAMP         1 TTCAATGCCTCTGTGCACTCAGACACGCCATCGGTCATCCGGGGAGATCTCATCAAACTGTTTTGTATCATCACTGTCGAAGGAGCGGCCCTGGATCCAGATGACATGGCCTTTGACGTG
PAMP ORF     1 F  N  A  S  V  H  S  D  T  P  S  V  I  R  G  D  L  I  K  L  F  C  I  I  T  V  E  G  A  A  L  D  P  D  D  M  A  F  D  V
FPRP ORF   690                            L        T                                  V        D        V
FPRP      2068 ..T..C...........T........T.TG..C....C.................GT....C......G.......G..C.....A.TG.....C................C..T..A

PAMP       121 TCCTGGTTTGCGGTGCACTCCTTCGGCCTGGACAAGGCTCCGGTCCTCCTGTCATCCCTGGATCGGAAGGGGATTGTGAACACAGCCGAGAGGGACTGGAAGAGTGGCCTCAGCCTGGAG
PAMP ORF    41 S  W  F  A  V  H  S  F  G  L  D  K  A  P  V  L  L  S  S  L  D  R  K  G  I  V  N  T  A  E  R  D  W  K  S  G  L  S  L  E
FPRP ORF   730                               I                          V     T     G  Q              T  V
FPRP      2188 ...........A..A.....T..T...T.............CA.......A..C...T....C........AG.C....CT....G.C...............AC.G...........

PAMP       241 CGCGTGAGCGTGCTGGAATTCTTGCTGCAAGTGCATGGCTCCGAGGACCAGGACTTTGGCAACTACTACTGCTCCGTGACTCCGTGGGTGAAGTCACCCACAGGTTCCTGGCAGAAGGAG
PAMP ORF    81 R  V  S  V  L  E  F  L  L  Q  V  H  G  S  E  D  Q  D  F  G  N  Y  Y  C  S  V  T  P  W  V  K  S  P  T  G  S  W  Q  K  E
FPRP ORF   770                                                                             R                          R
FPRP      2308 ..A................T..............A....T....................T..T..T........C.......G......A..T.............G...A

PAMP       361 GCAGAGATCCAGTCCAAGCCCATCTTTATAACTGTGAAGATGGATG     1537 bp     TGCTGAACGCCTTCAAGTACCCGCTGCTGATCGGCGTCGGCCTGTCCACGGTCATCGGGC
PAMP ORF   121 A  E  I  Q  S  K  P  I  F  I  T  V  K  M  D           intron      V  L  N  A  F  K  Y  P  L  L  I  G  V  G  L  S  T  V  I  G
FPRP ORF   810          H     R                                                                                  ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
FPRP      2428 ..C........C....G.................               .............................G..........A..........

PAMP       467 TCCTGTCCTGCCTCATCGGGTACTGCAGCTCCCACTGGTGCTGTAAGAAGGAGGTCCAGGAGACCAGGCGGGAGCGCCGCCGGCTCATGTCCATGGAGATGGACTAGgcggggccgggag
PAMP ORF   157 L  L  S  C  L  I  G  Y  C  S  S  H  W  C  C  K  K  E  V  Q  E  T  R  R  E  R  R  R  L  M  S  M  E  M  D  *
FPRP ORF   846 ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾                              R
FPRP      2534 ................T...........T.....T....................G.G......GC.T...........A....................T..Agc--agtt....

PAMP       587 gactggggcagccggacggccgaggaacaatttggg-cgagaagaggacagtgagcttttaacgcgaagcgtgttactctaaaaaccagtcctctctaatctcaggtgggacctggcgct
FPRP      2654 .gaca.a.g.a.gttgta.ga.ca.tgggg.gg...gt...........tc....a.....caa.cg..t.......a..-......g.....................tca.....

PAMP       707 ctctcttttctgcatgtcaagttctgagcgcggagatgtttaccagcacacggctcttcttcccacggcactttctgagcgaacaatcgagtgtgtgttctcccaacggcggctttttaa
FPRP      2774 ....................c.......c..................................................tatgtaacaatc.a......gtct..cc.cc.ccgc.gaagc

PAMP       827 tggttaaccttcgtctaaatttgttctcctactggcgtagagaaaaaaaaaaaaaaaaa
FPRP      2894 ..t....tgg.taa.ccccg.ctaat.ag.tt.ct.c...ca
```

Figure 1. Comparison of the DNA sequence of the PAMP cDNA insert and the overlapping region of the rat prostaglandin $F_{2\alpha}$ receptor regulator protein (FPRP). Open reading frames are translated below the DNA sequence for the PAMP and above the FPRP DNA sequence. Sequences are identical except where indicated. Coding regions are shown in capital letters and non-coding regions are shown in lower case letters. The amino acids predicted to be included in the transmembrane region are underlined. Dashes in the nucleotide sequence indicate deletions.

It was hypothesized that divergence in the two regions of sequence in the 3'

untranslated region may be due to exon splicing where the rat uses one exon and the pig

another for each region. To test this hypothesis, two primers encompassing most of the

coding region and the first area of divergence in the 3' untranslated region were used to

amplify genomic DNA by using the polymerase chain reaction (PCR). A 2114 bp frag-

ment was cloned and fully sequenced as shown in figure 2. It matched existing PAMP

cDNA sequence 100% and revealed one intron in the coding region of the cDNA.

```
   1 CTCCTGTCAT CCCTGGATCG GAAGGGGATT GTGAACACAG CCGAGAGGGA CTGGAAGAGT
  61 GGCCTCAGCC TGGAGCGCGT GAGCGTGCTG GAATTCTTGC TGCAAGTGCA TGGCTCCGAG
 121 GACCAGGACT TTGGCAACTA CTACTGCTCC GTGACTCCGT GGGTGAAGTC ACCCACAGGT
 181 TCCTGGCAGA AGGAGGCAGA GATCCAGTCC AAGCCCATCT TTATAACTGT GAAGATGGAT
 241 Ggtaagaatg acacacagat cactgtctca gtgtttgggg aatgtcttct ctctgtgctc
 301 tgctgccact gacgggatcc aggtccccac cctttgagga atgtgcctgc tttctctcta
 361 gacctggccc gggcctggac ccagaaggag cacatgcagc ccttgctgtt ttcccagctc
 421 tgatatagcc tgtgtgctgg aggcaccatt gggaaatgat ttttaagact cactggaaag
 481 attttcgata ctttctataa attctgtgcc ctcccacttt gccgtgtcct agggttggca
 541 caggtctgct gggcaagggc agagaatagc atcgagagag caggtccagg cctccaggag
 601 gggctgcggg gtgggttcct gccctcttgc cgtcctactg gttctgctct tggaaaatgg
 661 gggtcccctg gtgtcactag cacagagggg gttcttcatg gaaccctgag atgggagggg
 721 gagcttccca ccttgttggt ccacactgac caccacagtc agcctaacta ggtggtcaga
 781 ggcccccccc cacccctctg ctccctgagc atcccagagt tgggggcagg ggtattcctg
 841 ggtgcataag agacatgtct tccccgcaga aagcatcaaa ggaaacagta attgaaaaaa
 901 ctcatggaaa gaattcaagt ccatgtccct ctgaggaggc tttgagtgtg tttccctgat
 961 gatttgtcca tttgggggag gctggaattg gagccccagc tcttcccccg atgacttagc
1021 ctttctaatt cttcatttcc tcttttgtaa aagggatgat aatctctcgg agggctgttg
1081 tgctaaatgt caggcaacaa gcacagtgct agcacccacc cagggcgtgc tggtcctttg
1141 ggggaaggga ttgtgggctt tggggggtgtg ggatctgaac agtgtttgtg cattcctgaa
1201 acttagtcgt ttatacttat ttgcatccta gagatttctt gctggaagtt actccctatg
1261 aaaacgcaga tttaccaaac aagtagggag tgattattca cttttgctaga taatttacta
1321 cagtgttact taaaatagct ccctagtacg cttcagttat ttgatgcatg gaattgattt
1381 acgtatgatt ttttttaaagg atgcatctgc tgctgaatgt gtggtgccct tatactgact
1441 tttaaaaatc aatctcctgc caaaaccta aaaagaagca agttgacatt caaagacatc
1501 gaaggaaatc ttttttttctg gttttttctt ttttcttttt tttttttttt ttttggcccc
1561 tccagagaac tgggtgggga gacatttgct catttcttgg cagctgcggc tgagccgctt
1621 ctgcctcaag cactgtgcta ggtgggtgct gagtacagga tgcatgaggt ccgattcccc
1681 gggggagacc tgccccagtg gagcggggca gcggtgctct tcctggaacc ccctcgcatc
1741 tctaaccggt cactttgcct ttgctctctc cctcccagTG CTGAACGCCT TCAAGTACCC
1801 GCTGCTGATC GGCGTCGGCC TGTCCACGGT CATCGGGCTC CTGTCCTGCC TCATCGGGTA
1861 CTGCAGCTCC CACTGGTGCT GTAAGAAGGA GGTCCAGGAG ACCAGGCGGG AGCGCCGCCG
1921 GCTCATGTCC ATGGAGATGG ACTAGGCGGG GCCGGGAGGA CTGGGGCAGC CGGACGGCCG
1981 AGGAACAATT TGGGCGAGAA GAGGACAGTG AGCTTTTAAC GCGAAGCGTG TTACTCTAAA
2041 AACCAGTCCT CTCTAATCTC AGGTGGGACC TGGCGCTCTC TCTTTTCTGC ATGTCAAGTT
2101 CTGAGCGCGG AGAT
```

Figure 2. Sequence of genomic PCR product. Underlined nucleotides are primers which were also used for reverse transcriptase-PCR. Bold capital letters indicate coding regions of the exon while normal capital letters indicate the 3' non-coding region of the exon.

Southern blotting using the PAMP cDNA fragment to make random primed probe shows
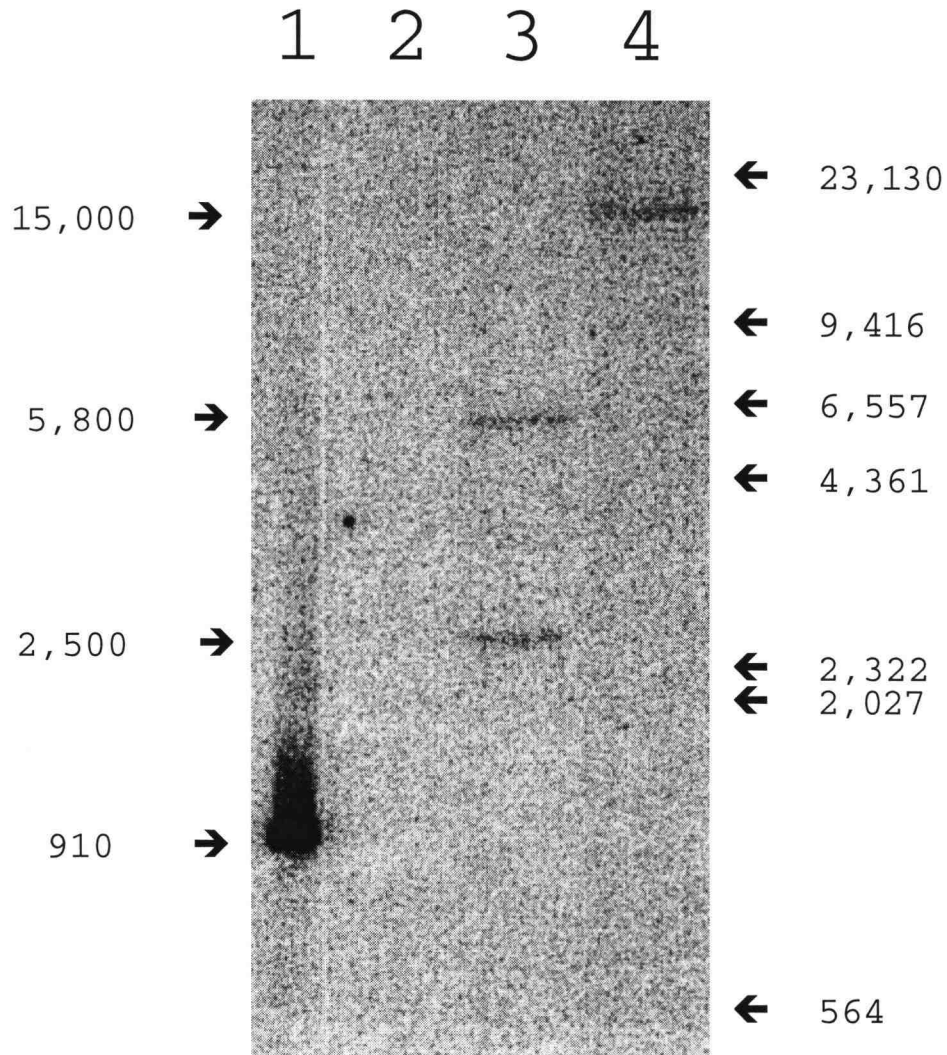this to be a single copy gene (figure 3).

Figure 3. Southern blot analysis of porcine genomic DNA. The 910 bp PAMP cDNA
insert was used to generate random labeled probes using ^{32}P. Twenty to thirty micrograms
of genomic DNA, isolated from porcine liver, was digested, electrophoresed in an agarose
gel, stained with ethidium bromide, photographed, and transferred to nylon membrane.
Five nanograms of PAMP cDNA insert was used as a positive control and a *Hind*III digest
of bacteriophage lambda DNA was used as a molecular weight standard. Arrows on the
right side of the figure show sizes and positions of molecular weight standards. Arrows
on the left side of the figure show sizes and positions of hybridization signals. Lane 1,
PAMP cDNA insert. Lane 2, Bacteriophage lambda molecular weight standard. Lane 3,
Porcine genomic DNA digested with *Bam*HI restriction endonuclease. Lane 4, Porcine
genomic DNA digested with *Hind*III restriction endonuclease.

Digestion of porcine genomic DNA with the restriction enzyme *Bam*HI yields two relatively small fragments of 2.5 and 5.8 kb while digestion with the restriction enzyme *Hind*III yields one large 15 kb fragment. This result is not surprising since sequence data from the cDNA and intron reveals 2 *Bam*HI sites but no *Hind*III sites. Sequence data is summarized in figure 4.
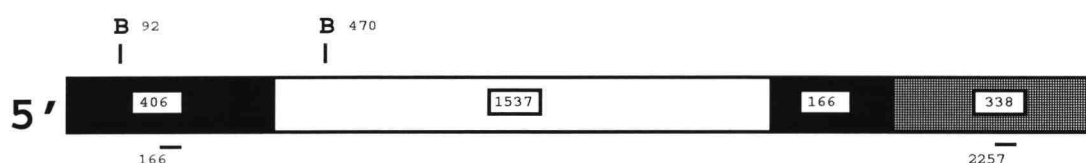

Figure 4. Schematic diagram showing the information obtained so far from the PAMP cDNA and genomic sequences. Black boxes represent exons, the white box represents the intron, and the checked box represents the 3′ non-coding region exons. Numbers inside each of the boxes indicate the number of nucleotides in that section. *Bam*HI sites and positions are indicated by B's. Lines under the boxes show the position of primers used for RT-PCR and amplification of the genomic segment. Numbers associated with the primers indicate the 5′ positon of the respective primer and starts from the first nucleotide of the cDNA sequence.

The amino acid sequence was analyzed using the Kyte and Doolittle method for predicting hydrophobic regions as shown in figure 5. A 23 amino acid region in this sequence is long enough to span the plasma membrane. This analysis agrees with Orlickey's prediction of a membrane spanning region for these amino acids (Orlickey and Nordeen, 1996). In addition, this protein was analyzed for secondary structure features as calculated using the Chou and Fasman rules. These predictions are also shown in figure 5.

Figure 5. Hydrophobicity plot for the predicted amino acid sequence of the PAMP. Hydrophobic regions are below the line. Cross-hatched area is the predicted transmembrane region. Secondary structure predictions based on the Chou and Fasman rules are shown at the bottom. α represents alpha helix, β represents beta sheet, and T represents turn predictions. Cys^{170} and Cys^{171}, which are shown in figure 8, are underlined.
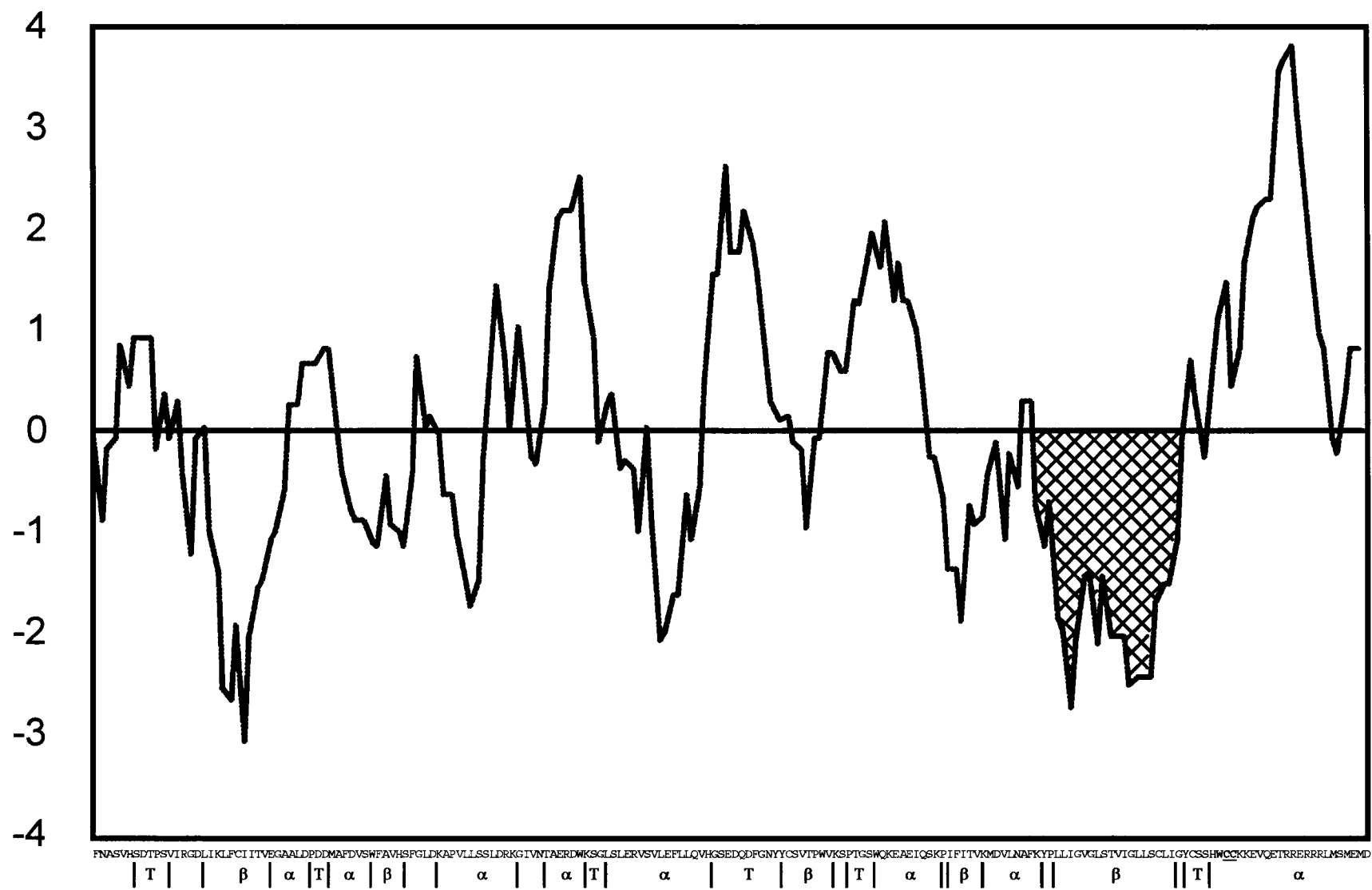
Figure 5

In order to verify the predicted amino acid size, a western blot analysis was performed with the *E. coli* fusion protein and both reduced and unreduced porcine adipocyte plasma membrane proteins from two different pigs (figure 6).
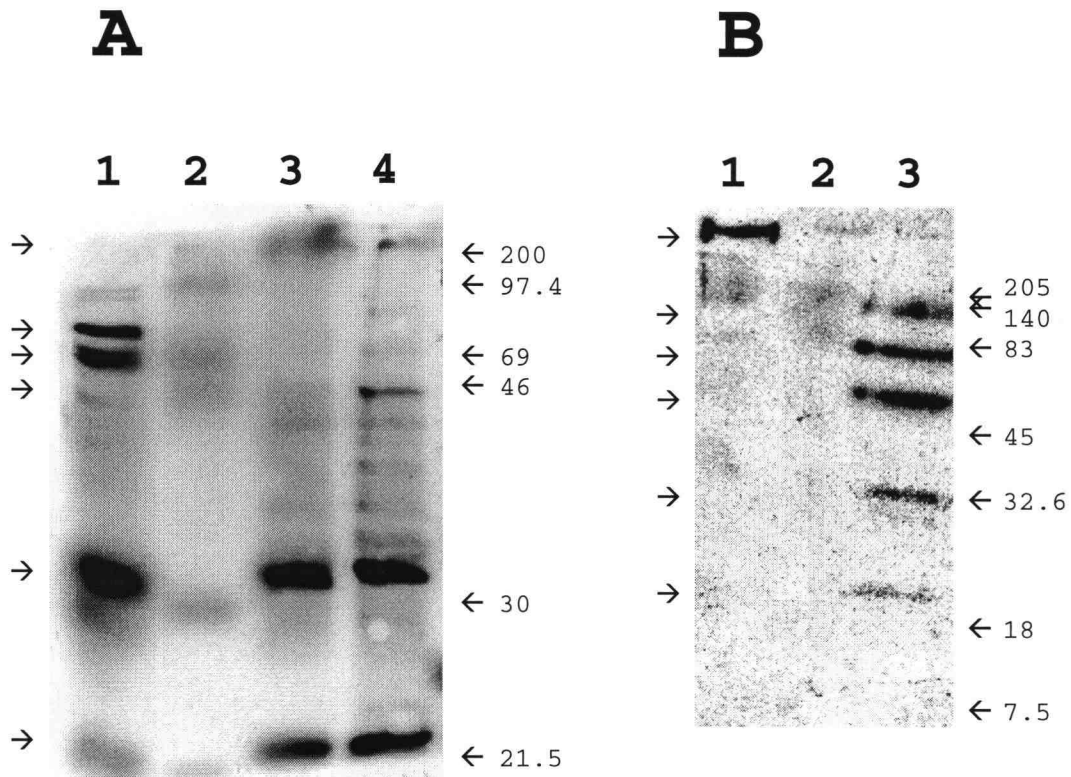

Figure 6. Western blots from two different porcine adipocyte plasma membrane samples using LA-1 primary antibody and enhanced chemilumenescent detection. Arrows on the right side of the blots show positions and values of molecular weight markers. Arrows on the left side of the blots show positive signals. Values for positive signals are listed in Table 1. A. 12% SDS-PAGE. Lane 1, *E. coli* fusion protein. Lane 2, Rainbow molecular weight marker. Lane 3, Reduced porcine adipocyte plasma membrane protein. Lane 4, Unreduced porcine adipocyte plasma membrane protein. B. 15% SDS-PAGE. Lane 1, Unreduced porcine adipocyte plasma membrane protein. Lane 2, Kaleidoscope molecular weight marker. Lane 3, Reduced porcine adipocyte plasma membrane protein.

Results of fragment analysis as shown in table 1 indicate that four *E. coli* fusion products are nearly the same size as the natural porcine protein with the smallest band at about 22.1 kD. Four bands were detected in the *E. coli* fusion product lanes measuring 22.1, 32.1, 68.0, and 77.8 kD. For the first pig in figure 6A, both reduced and unreduced preparations revealed 3 or 4 bands with the highest signal intensity in both cases at 22.5 or 22.9 and 32.1 kD. The second pig, in figure 6B, showed only one band in the unreduced lane which was well above the highest molecular weight marker and five bands in the reduced lane ranging from 21.0 to 172.4 kD.

TABLE 1. Molecular weights (kD) of bands in western gels from figure 6.

	Figure 6A		Figure 6B		
E. coli fusion protein	Porcine APM unreduced	Porcine APM reduced	Porcine APM unreduced	Porcine APM reduced	Porcine APM averages
	251.5	243.4	2092		
				172.4	172.4
77.8				78.2	78.2
68.0		46.0		55.1	50.5
32.1	32.1	32.1		32.8	32.3
22.1	22.5	22.9		21.0	22.1

APM = Adipocyte Plasma Membrane

A primary cell culture system has been developed which allows undifferentiated cells from 1 day old pigs to grow and differentiate under appropriate conditions (Akanbi et al., 1994). The expression of this protein was examined by a reverse transcriptase-PCR assay (RT-PCR). Total RNA was isolated from cultured cells every day over a ten day period. The thermostable enzyme Tth was used to make the first strand of DNA since this enzyme has reverse transcriptase activity in the presence of Mn^{2+}. Two aliquots of this

reaction were then used as template for a PCR reaction using Taq polymerase. The PCR product generated from an mRNA template was predicted to be 573 bp long. These primers encompass a 1537 bp intron so PCR products from genomic template would be 2114 bp.

As seen in figure 7, this message appears to be expressed at low levels throughout the growth and differentiation of the cells as at least one reaction is positive for each day. Production of the message appears to peak at 6-7 days as these samples gave positive signals in both reactions. This result was confirmed in one other trial where about 50-fold less total RNA was used as template and positive signals were seen only at 8 days of culture.

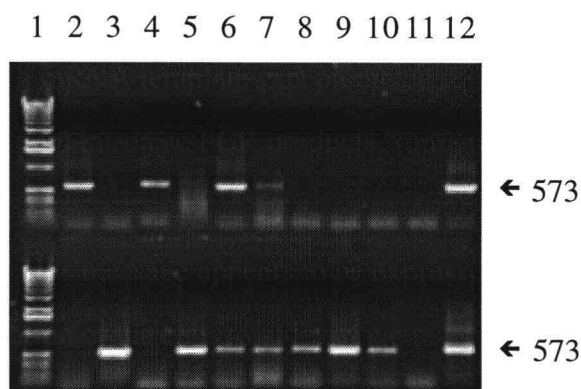1 2 3 4 5 6 7 8 9 10 11 12

Figure 7. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) for daily RNA samples of 1 day old pig stromal-vascular primary cell culture separated on 1% agarose. Each reverse transcriptase reaction was amplified in two separate PCR's. Positive reactions are indicated by the arrows on the right which designate the 573 bp product for each set of reactions. Lane 1, 1 Kb ladder (Gibco BRL). Lane 2, Day 2. Lane 3, Day 3. Lane 4, Day 4. Lane 5, Day 5. Lane 6, Day 6. Lane 7, Day 7. Lane 8, Day 8. Lane 9, Day 9. Lane 10, Day 10. Lane 11, Negative control. Lane 12, PAMP cDNA positive control.

DISCUSSION

Although the PAMP sequence represents only one-sixth of the length of the rat FPRP, it is 80% similar to the rat FPRP sequence in the overlapping segment. This sequence overlaps the last 192 amino acids of the 879 amino acid FPRP and 295 nucleotides of the 3' untranslated region. It would be reasonable to predict that the PAMP sequence is a partial sequence of a much larger protein which is homologous to the rat FPRP. However, while this is probably a partial sequence, the western data suggest that the amino acid sequence is nearly complete.

The *E. coli* fusion protein was generated by inserting a cDNA sequence into the ß-galactosidase gene of the λZapII expression vector. Transcription and translation begin at the start of the ß-galactosidase gene and continue through the inserted cDNA generating a fused protein consisting of 43 amino acids from the ß-galactosidase gene and, in this case, 192 amino acids of the PAMP gene. This protein is predicted to be 25.5 kD with no major modifications. As seen in figure 6A, a band near the predicted size of 25.5 kD (22.1 kD) was seen but three other bands were also seen. Coincidentally, protein from porcine adipocyte plasma membrane also revealed similar sized bands as shown in Table 1. The four bands from *E. coli* are most likely multimeric forms of the 22.1 kD protein. Since these four bands are nearly the same size as the four bands in the porcine tissue, this suggests that the native protein is also detected as multimeric forms of the 22.1 kD unit or a homotetrameric protein. The 172.4 kD band is probably a conformational variant and not a multimeric form since there are no bands between 78.2 and 172.4 kD. Oligomerization is unusual for an *E. coli* fusion protein since eukaryotic proteins are generally unmodified in a prokaryotic host. The presence of protein oligomers in this cloning system suggests spontaneous disulfide bond formation between monomers to form the homotetrameric protein.

The apparent oligomerization of the PAMP fusion protein is highly resistent to reduction, even with high levels of DTT. This is consistent with cysteine bonds located near or in the plasma membrane spanning region where they are less accessible to reducing agents. It is theorized that hydrophobic membrane spanning regions are naturally attracted to each other which brings the C-terminal alpha helix of each monomer together. In the fusion protein helix, very close to the hydrophobic region are two cysteine residues, Cys^{170} and Cys^{171}. When plotted on a helical wheel diagram as shown in figure 8, these cysteines are 100° apart which may allow four monomers to crosslink.
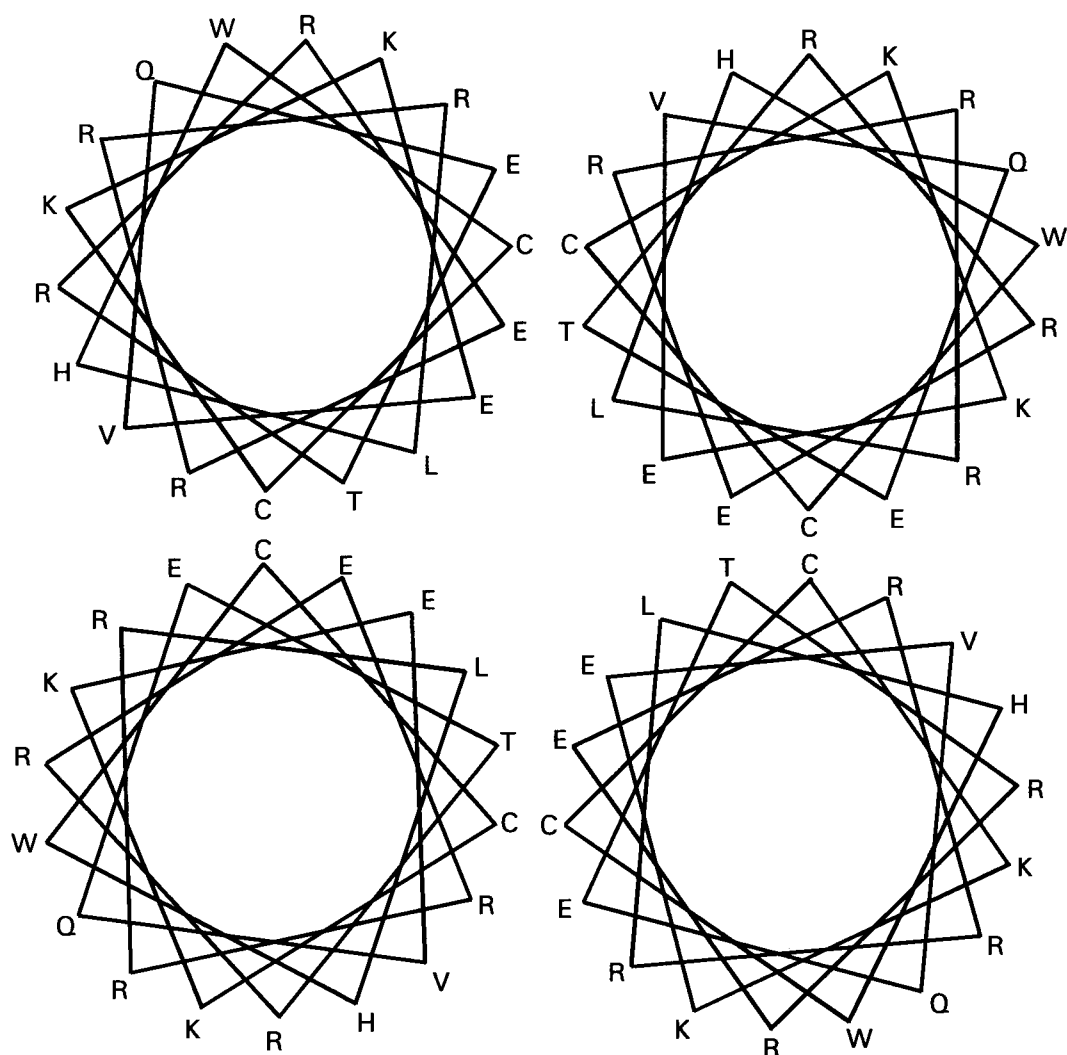
Figure 8. Helical wheel diagram for amino acids 168 through 185 which are in the car-
boxyl terminal alpha helix as predicted by the Chou and Fasman rules for secondary
structure analysis. Four identical helices can be arranged such that Cys[170] of one helix is
near Cys[171] of the adjacent helix thus allowing the four monomeric units to crosslink and
form a homotetrameric protein.

Previous western analyses indicated a single band at 64 kD (Killefer and Hu, 1990b). It is likely that this was the result of reduction of proteins with β-mercaptoethanol which is a milder reducing agent than DTT. Also, Killefer and Hu (1990b) used an alkaline phosphatase detection method which is less sensitive than the enhanced chemilumenescent detection method.

An interesting aspect of this theory is allelic variation which appears to be indicated by the results in figures 6A and 6B. Although plasma membrane preparations from two pigs are treated similarly, one is nearly fully reduced to monomeric and dimeric units, even with no reducing agent, while the other is mostly trimeric and tetrameric units. Subsequent attempts to reduce the protein from the second pig with sodium borohydride and 8M urea did not improve reduction of this sample (data not shown). It is possible that genetic variation in the region of proposed disulfide bond formation may make this area more susceptible to reduction in the case of the sample shown in figure 6A. Samples from two more pigs were analyzed and the results were similar to the sample in figure 6B (data not shown) where most of the protein was in tetrameric and trimeric forms and very little was reduced to dimeric or monomeric forms. Unfortunately, this possibility can not be investigated since DNA samples were not retained from these pigs. Further evidence for tetramer formation is indicated by the unreduced porcine proteins which indicate a protein larger than the 200 and 205 kD molecular weight markers. In the 12% polyacrylamide gel, this protein migrates close to the 200 kD marker but in the 15% polyacrylamide gel, it barely enters the gel. Assuming that disulfide bonds form as described by figure 8, this result is not unexpected since the unreduced proteins would be in a globular form instead of a linear form. The globular form would be sterically hindered from migrating in the gel, especially in the case of the 15% gel which is best suited for separating proteins in the 1-80 kD range. Since the western data for the fusion protein agrees closely with the native protein, it is probable that the cDNA, while lacking some 5′ sequence, is nearly complete. Clearly, from the western data, the FPRP is much larger than the porcine unknown pro-

tein. One explanation for the size difference is alternative splicing of the gene. This possibility is supported by genomic sequence of the 5' end of the rat FPRP gene which has an intron 17 amino acids after the start codon (Orlickey et al., 1996). If the gene is arranged similarly in the pig, this exon could be used in the porcine protein to start the protein and provide a membrane protein signal but still produce a smaller protein by splicing closer to the 3' end.

It was noted that there are regions of 49 and 84 nucleotides each with 33% identity to rat FPRP sequence in the 3' untranslated region. The first region is immediately downstream from the stop codon. These regions of low similarity between the two species may indicate different exon usage which would support the alternative splicing hypothesis. Two primers encompassing the region immediately after the stop codon were used to PCR amplify the porcine genomic fragment. Subsequent sequence analysis, however, revealed that this region is not a separate exon and therefore, this dissimilar region is most likely due to natural divergence and not alternative splicing. The other dissimilar region has not been sequenced from a genomic clone.

It is interesting that the second dissimilar region of 84 nucleotides is immediately prior to the poly A sequence in the PAMP cDNA. This placement of the poly A tail is far different from that of the rat FPRP which is 2847 nucleotides further downstream. Also, Orlickey's analysis (Orlickey and Nordeen, 1996) indicates seven de-stabilization signals (ATTTA) and an inflammatory mediator sequence (TTATTTTAT) which would be absent in the PAMP cDNA if the poly A tail placement is accurate. Unfortunately, the PAMP cDNA does not have the highly conserved poly A addition signal sequence (AATAAA) anywhere in the 3' untranslated region so the location of the tail may be due to a mispriming event during cDNA synthesis. On the other hand, if the poly A addition signal has somehow been lost during cDNA synthesis but the 3' untranslated region is similar in size, it is possible that the areas of difference and the alternate placement of the poly A tail may indicate a different regulatory mechanism for this protein.

Despite the ambiguities concerning the amino acid and nucleotide sequence of this gene, the transcription of this gene can still be studied in a serum-free cell culture system as demonstrated by the RT-PCR experiments. This assay has successfully amplified 10^{-15} g of plasmid containing the PAMP cDNA insert as a positive control which is less than 1000 gene copies at the start. Although RT-PCR is not a strictly quantitative assay, the results indicate that this message is a very low abundance message. Since cells in primary culture reach confluence after 2 or 3 days, the peak production of this message is 3 to 5 days post-confluence which is a time when most cells are differentiated and actively accumulating lipids. This model system can be used to determine which growth factors may influence the transcription of this message.

Western blot analysis of genetically lean, obese, and contemporary pigs indicated that the protein was not present in obese pigs (Killefer and Hu, 1990a). The RT-PCR assay was used to determine if this difference was due to transcriptional regulation of the mRNA. In one assay, both lean and obese pigs produced a positive signal. Subsequent sequencing of the PCR product from the obese pig showed no sequence differences from the lean pig. This does not invalidate the Western results since regulation of the protein may be at the translational stage. Also, the entire coding region is not assayed so there may be a nonsense mutation prior to the region which is amplified which would also prevent production of the protein.

Since the predicted size and conformation of PAMP is so different from FPRP, it is not clear if they perform the same function. The FPRP was found only in reproductive tissues, heart, and lung (Orlickey and Nordeen, 1996). Examination of cytohistochemical slides show evidence of FPRP protein in rat adipose cells (D. Orlickey, personal communication). By comparison, the PAMP was found in adipose but not heart or lung and reproductive tissue was not examined (Killefer and Hu, 1990b). Deletion mutations of the rat FPRP cDNA seem to indicate that the inhibitory activity of the protein is on the carboxyl side of the transmembrane region (Orlickey, 1996) which is highly conserved between the

rat and the pig. Tissue specific alternative splicing of the gamma subunit of the human mitochondrial ATP synthase has been found to occur (Matsuda et al., 1993) which indicates that this proposed model has a precedent.

It is clear that further experimentation must be done to ascertain the function of the PAMP and its significance in adipocytes. Detection of multiple forms of the mRNA from different tissues would confirm an alternative splicing mechanism. Sequencing of the genomic DNA in the region of the gene would provide information for the design of primers for RT-PCR amplification of low copy number alternative messages. Site-directed mutagenesis studies could ascertain which cysteines are used to form disulfide bonds. Finally, assays for mRNA expression in primary cell cultures could yield further insights to the function of this protein and its possible role in adipocyte development.

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APPENDICES

Appendix A: Development of RT-PCR Assay

There are several unusual features in the methods used to assay mRNA by the RT-PCR described in this report. This section will explain the experiments conducted to determine the optimal parameters for this assay.

The first step was to determine the optimal buffer conditions for the PCR using the two primers reported previously and the PAMP cDNA plasmid. The Optimizer Kit from Invitrogen was used to show that amplification occurred only at pH 9.0, but was successful over a range of Mg^{2+} concentrations from 1.0 to 3.5 mM.

Next, several attempts were made to assay adipose RNA using the cDNA Cycle kit also from Invitrogen. This kit uses oligo-dT and random primers and the Murine Moloney Leukemia Virus (MMLV) reverse transcriptase. In addition, the PAMP specific primer was used. No amplifications occurred using this kit. It was theorized that mRNA secondary structure might be interfering with the reverse transcription reaction so the thermostable enzyme Tth was used to synthesize cDNA at RNA-denaturing temperatures. This enzyme has reverse transcriptase activity in the presence of Mn^{2+} and DNA polymerase activity in the presence of Mg^{2+}.

The ideal procedure is to complete the reverse transcriptase reaction and then add EGTA to bind the Mn^{2+} and the rest of the PCR components to amplify the entire reverse transcriptase reaction. However, as the conditions for the PCR had been optimized for the Taq enzyme, it was decided to add a reaction mix which included a buffer at the correct pH, EGTA, and Taq. This approach gave a weak positive result. A portion of a 35 cycle PCR reaction was then re-amplified in a fresh PCR reaction for an additional 35 cycles which gave a strong positive result. The next experiment added the PCR reaction to the RT reaction but amplified for 60 cycles instead of a normal 35 cycles.   This failed to yield amplification products. Again a portion of the reaction was successfully amplified in a fresh PCR reaction mix. This suggested that the buffer from the RT reaction was interfer-

ing with the pH, so a portion of the RT reaction was aliquoted to a fresh tube with the PCR components. Cycling for 35 cycles was insufficient but 60 to 65 cycles gave strong positive results. Since the PCR is subjected to extra cycles, 50% more Taq is used to ensure that there is an adequate amount of enzyme for the later cycles.

Therefore, for this assay, the following conditions are critical for success: 1. Tth enzyme should be used for the RT reaction. 2. The PCR with Taq enzyme needs a buffer with a pH of 9.0. 3. The PCR needs to cycle for 60 to 65 cycles.

It should be noted that the use of MMLV or other reverse transcriptase enzymes from viruses were not re-investigated. It seems that the thermostable enzyme Tth should be a better choice in most cases since extension at the higher temperatures should be less hindered by secondary structure in the RNA and thus proceed more processively than at the lower temperatures required for the viral enzymes. Also, the exact number of cycles necessary for amplification was not rigorously tested since reactions can be automatically run overnight so time is not a factor. Thus, there was no need to investigate the consequences of using a number of cycles between 35 and 65, especially since non-specific bands were not routinely produced with the extra cycles.

Appendix B: RT-PCR Analysis of Mature Porcine Tissue RNA

In Killefer and Hu (1990b), the PAMP protein is shown to be tissue specific based on the results of an immunoblot using several different tissue plasma membrane preparations. An attempt was made to extend these results by determining the level of mRNA expression for this protein in each of the tissues. RNA was isolated from tissue samples collected from each of three freshly slaughtered pigs. Electrophoresis and blotting followed by probing with randomly labeled radioactive DNA of both total RNA (20 micrograms) and mRNA (3 micrograms) failed to yield any signals. Therefore, new samples were collected and subjected to RT-PCR analysis. As seen in table 2, no tissue gave positive results for all three pigs, including adipose.

Table 2. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) for ten different tissues from each of three pigs. + indicates that a PCR product of 573 bp was formed for this tissue.

SAMPLE	PIG 1	PIG 2	PIG 3
ADIPOSE	+	-	-
BRAIN	+	-	-
HEART	+	-	+
KIDNEY	+	-	+
LARGE INTESTINE	-	-	+
LIVER	-	+	-
MUSCLE	+	-	-
SMALL INTESTINE	-	+	-
SPLEEN	+	+	-
STOMACH	-	-	-
NEGATIVE CONTROL	-	-	-
POSITIVE CONTROL	+	+	+

Therefore, it is difficult to reach any conclusions based on these results. These results do not necessarily invalidate the previous western results as this RT-PCR assay could detect alternatively spliced mRNA variants present in other tissues. Although extreme care was taken during isolation of tissues, it is also possible that adipose tissue was contaminating some of the tissue samples since adipose tissue is present in many depots throughout the body. This contamination could be the source of positive signals instead of the mRNA from the intended tissue. One method to improve the experimental design would be to assay in parallel for a relatively rare message common to all tissues which would demonstrate the quality and the purity of the RNA for each tissue sample. Information about the 5′ end of the mRNA would lead to the design of better primers which might distinguish alternatively spliced variants. Finally, another parallel RT-PCR assay could be performed to detect another adipose specific gene such as adipocyte lipid binding protein to detect the contamination of desired tissues with adipose cells.

Appendix C: Full-length cDNA Search

Several attempts were made to recover a more complete clone from the original cDNA library. Primers specific to the PAMP sequence were used in a PCR with an oligo-dT primer, M13 primers, and random primers to detect clones which were longer than the original PAMP clone. However, no products of a larger size were detected from this cDNA library. This result seemed to indicate that no additional full length clones with this sequence are in the cDNA library. Future attempts should use fresh mRNA from mature porcine adipose tissue or day 6-8 of primary cell culture. As mentioned previously, information from genomic sequence may aid in the design of a primer to capture more of the 5'end.