Molecular Cloning, Expression, and Chromosomal Assignment of Sarcolemmal-associated Proteins

A FAMILY OF ACIDIC AMPHIPATHIC α-HELICAL PROTEINS ASSOCIATED WITH THE MEMBRANE

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Two overlapping cDNAs encoding a novel sarcolemmal associated protein (SLAP) were isolated from a cardiac cDNA expression library by immunoscreening with anti-sarcolemmal antibodies. Further characterization of these clones showed that they belonged to a family of related cDNAs that potentially encode polypeptides of 37, 46, and 74 kDa designated SLAP1, SLAP2, and SLAP3, respectively. The SLAP3 transcript was ubiquitously expressed, whereas SLAP1 and SLAP2 transcripts were predominantly expressed in cardiac, soleus, and smooth muscle. SLAP was encoded by a single gene that mapped to chromosome 3p14.3–21.2, and the various transcripts are likely generated by alternative splicing. The primary structure of SLAP predicted that it would have large regions of coiled-coil structure including an 11-heptad acidic amphipathic α-helical segment. The carboxyl-terminal region of the SLAP proteins was predicted to have a transmembrane domain, although there was no discernible signal sequence. SLAPs could only be solubilized from cardiac membrane with detergents suggesting that they were integral membrane proteins. Subcellular distribution studies showed that MYC epitope-tagged SLAP localized to regions of juxtaposition between neighboring cell membranes although an intracellular pool of the protein was also present in cells undergoing apparent cleavage. Immunohistochemical localization of SLAP in cardiac muscle revealed that SLAP associated with the sarcolemma and also displayed a reticular pattern of staining that resembled the transverse tubules and the sarcoplasmic reticulum. The SLAPs define a new family of tail-anchored membrane proteins that exhibit tissue-specific expression and are uniquely situated to serve a variety of roles through their coiled-coil motifs.

In muscle cells, the membrane, also referred to as the sarcolemma, invaginates at discrete intervals to form the transverse tubules that extend into the cell interior where they physically associate with the intracellular membranes of the sarcoplasmic reticulum (1). This specialized architecture of the membrane allows for the efficient coupling of membrane excitability to the contractile response in muscle cells. The depolarization of the sarcolemma leads to the activation of voltage sensors concentrated in the transverse tubules which trigger calcium release from the sarcoplasmic reticulum and initiate muscle contraction (2–5). Although there are major differences in membrane composition between divergent cells, more subtle yet critical differences in molecular composition of the membrane have also been noted in cells originating from a common lineage. For example, the molecular components of cardiac sarcolemma have undergone significant alterations to adapt to the functional requirements of the cardiac cell when compared with its skeletal muscle counterpart. Unlike skeletal muscle, the heart cells require external calcium for excitation-contraction coupling. The cardiac voltage-gated calcium channel is adapted to allow calcium entry across the sarcolemmal membrane which then serves as the trigger for calcium release from intracellular stores of the sarcoplasmic reticulum (6). The calcium that enters the cardiac cell is then extruded via an active sodium-calcium exchange mechanism that has been shown to be concentrated in the transverse tubules through association with ankyrin (7, 8).

Although the advent of molecular biological techniques has resulted in a wealth of information about membrane proteins with measurable biochemical activities, some of the minor components that may participate in major functions remain to be defined. For example, syntaxin, a neural tail-anchored membrane protein, has been shown to bind to the N-type calcium channel and localize it to the site of vesicle release (9, 10). This allows for rapid exocytosis of synaptic vesicles by localizing sites of calcium influx to where synaptic vesicles are docked. In an effort to understand some of the molecular components of the heart cell membrane, we had raised and characterized antibodies to highly purified sarcolemma (11). By using these antibodies as probes to screen a cardiac cDNA expression library, two cDNAs were isolated that encoded a novel family of sarcolemmal associated proteins which we have termed SLAPs.1 The SLAP proteins were predicted to contain an extended coiled-coil structure and a carboxyl-terminal transmembrane domain. Subcellular localization of SLAP reveals that they can reside not only in the cell membrane but also in intracellular compartments. Furthermore, the expression of

1 The abbreviations used are: SLAP, sarcolemmal associated protein; DAPI, 4′,6-diamidino-2-phenylindole; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; kb, kilobase pair(s); FISH, fluorescence in situ hybridization; nt, nucleotides; PBS, phosphate-buffered saline; bp, base pair(s); MOPS, 4-morpholinepropanesulfonic acid.
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SLAPs in diverse tissues imply that these proteins may serve important roles in protein-protein interactions through their coiled-coil motifs in membranes from different cell types.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning of SLAPs—Total RNA was isolated from rabbit heart by the guanidine thiocyanate method (12), and the mRNA was then affinity purified by oligo(dT)-cellulose chromatography (13). A rabbit heart cDNA expression library was constructed using λ gt-11 phage vector according to the manufacturer’s instructions (Amersham Corp.). The cDNA synthesis was primed with oligo(dT). The final library contained $1 \times 10^8$ plaques of which $5 \times 10^5$ plaques were screened with antisera raised to highly purified canine cardiac sarcolemma (11). Only two positive clones (LD1,LD2) were detected by screening with the antisera. The two positive clones were shotguned into PTZ19R (Pharmacia Biotech Inc.). A second λ gt-11 library ($4 \times 10^9$ plaque-forming units) was constructed from rabbit heart mRNA, but cDNA synthesis was primed with random hexamers (Life Technologies, Inc.). 250,000 plaques of this library were screened with a 366-bp AvaI/EcoRI insert from LD1 that was labeled with [α-32P]dCTP by the random primer method (Fig. 1A). Three positive clones from a tertiary screen (R3a, R4b, and R4t) were subcloned into Bluescript II KS (Stratagene). A kit for rapid amplification of cDNA ends was used to identify the 5′ ends of the cDNA synthesis clones (Life Technologies, Inc.). The reverse transcriptase reaction was primed with an oligonucleotide (nt 2150–2133 of SLAP3), and another nested oligonucleotide (nt 2113–2093 of SLAP3) was isolated by screening a human cardiac cDNA library (4,5) with a 1.1-kb EcoRI/XbaI fragment from the rabbit LD1 clone as a probe (Fig. 1A).

**Sequencing SLAP Clones—**ExoIII/S1 nuclease deletions (Promega) of the positive clones were made. Single-stranded DNA was isolated from deletions of LD1 and LD2, and double-stranded plasmid DNA was isolated (Qiagen) from the deletions of the other clones (R3a, R4b, and R4t). The deletions were sequenced on both strands by the dyeoxy chain terminator method (14) using Sequenase (Amersham Corp.). E. a. sequence not adequately covered by deletions were resolved by sequencing with specific internal primers (University of Ottawa Biotechnology Institute). Sequence analysis was performed using the GeneScan nucleotide computer program, BLAST WWW server (15), PSORT E-mail server (16), Sequid (University of Kansas), and the Coils analysis program (17).

**Northern Blot Hybridization—**Rabbit tissues (brain, heart, soleus (slow twitch), and longitudinus dorsi (fast twitch)) were excised and immediately neutralized with 1M Tris (pH 9.5) until the pH was 8.0. Subcellular Fractionation and Solubilization—Crude and purified rabbit cardiac sarcolemma membranes were isolated as described previously (20). Membrane proteins were subjected to electrophoresis in a highly porous SDS-PAGE system which permits rapid and efficient transfer of proteins to nitrocellulose while maintaining a broad degree of resolution (21). The proteins, resolved on gels, were electrophoretically transferred to nitrocellulose membranes (22). The affinity purified guinea pig anti-(SLAP) antibodies were used at a dilution of 1:5000, and the reaction developed with alkaline phosphatase-conjugated anti-guinea pig IgG (Jackson Immunoresearch Laboratories). Membrane proteins were subjected to electrophoresis in a 10% SDS-PAGE gel followed by autoradiography.

**Plasmid Construction and in Vitro Transcription/Translation—**A MYC-tagged SLAP protein was constructed by subcloning BglII/XhoI-digested SLAP3 restriction fragment (encoded all of SLAP1, SLAP2, and from amino acid 184 of SLAP3) into the six repeat human MYC epitope of Bluescript KS + MYC-tagged vector (23). The cDNA encoding the fusion protein was excised with the appropriate restriction enzymes (KpnI/XbaI) and subcloned into pcDNA3 (Invitrogen). The Slap coding sequence including the first ATG was PCR-amplified with IVTRO (5′-ggcatgagtcaCTTTCAGAGCAGATAC-3′ and TM3′ (5′-ggaattcccCTTTCAGACGAGATAC-3′) using Slap3 construct as a template. The PCR product was digested with Xhol/EcoRI and cloned into Xhol/EcoRI-digested pBluescript II KS+ to make the ATG1 plasmid. The ATG2 plasmid (which only contained the second ATG) was constructed by subcloning the KpnI/EcoRI restriction fragment of SLAP3 into KpnI/EcoRI-digested pBluescript II KS+. The two SLAP plasmids were linearized with full-length SmaI and then cloned into SmaI-digested lambda vector. One μg of RNA was used to program a rabbit reticulocyte lysate (Promega) in the presence of [23S]methionine. Diethylpyrocarbonate-treated water and a luciferase transcript were used as negative and positive controls. Equal aliquots of each reaction were analyzed by SDS-PAGE followed by autoradiography.

**Cell Culture and Expression of 6MYC-Tagged Recombinant SLAP Protein—**P19 embryonal carcinoma cells were cultured in a minimum essential medium supplemented with 7.5% donor bovine serum and 2.5% fetal bovine serum (Cansera). P19 cells were transfected with the 6MYC-SLAP plasmid DNA, and stable transfectants were selected in G418 as described previously (24). Immunofluorescence studies of cultured P19 cells was performed as described previously (25). Anti-human MYC monoclonal antibody 9E10 was diluted 1:50 in 10 ml PBS containing 0.3% Triton X-100 (26). CY3-conjugated sheep anti-mouse secondary antiseraum (Sigma) was diluted 1:100 in 10 ml PBS, 0.3% Triton X-100. The cells were examined by using an upright Leica confocal scanning laser microscope equipped with a 55-milliwatt krypton/argon air-cooled laser and a 63 × Plan Apo oil immersion lens. The negative controls for immunofluorescence studies was P19 cells mock-transfected P19 cells stained with empty pcDNA3 vector.

**Immunohistochemistry—**After decapsulation, rat hearts were removed and immediately frozen in powdered dry ice. Sections were cut at a thickness of 4 μm on a cryostat and then fixed for 5 min using a fixative consisting of 4% (w/v) paraformaldehyde and 12% (v/v) saturated picric acid in 160 mM sodium phosphate buffer (pH 6.9). After rinsing, the sections were incubated in either a 1:400 dilution of rabbit antisera was then passed three times over the SLAP fusion column. The column was washed extensively with 10 ml MOPS buffer (pH 7.4) and then with Tritis-buffered saline containing 0.05% Tween (TBS-T). The specifically bound antibodies were eluted with 0.1 ml glycine (pH 2.5) and immediately neutralized with 0.1 volume of 1 M MOPS buffer (pH 6.0).
anti-SLAP antiserum or a 1:100 dilution of affinity purified rabbit anti-dystrophin antiserum raised to the carboxyl terminus of dystrophin (27) overnight at 4 °C. Primary antibodies were applied to the slide-mounted sections in 10 mM sodium phosphate buffer containing 0.3% Triton X-100. Sections were rinsed in PBS and then incubated with a 1:25 dilution of fluorescein isothiocyanate-labeled donkey anti-rabbit (Amersham Corp.). Slides were coverslipped using a mounting medium of PBS containing phenylaminediamine (0.1 mM) and 90% glycerol and examined using a Zeiss Axioplan microscope with a 100 × oil immersion lens.

Southern Blot Hybridization—Rabbit blood genomic DNA was purified as described previously (18). 20 μg of purified genomic DNA was digested with the indicated restriction enzymes and size-fractionated on a 0.75% TBE agarose gel. After size fractionation the DNA was denatured with alkali, neutralized, and transferred to Magna nylon membranes (MSI). A 366-bp EcoRI/AvaI fragment from LD1 was radio-labeled with [α-32P]dCTP by the random primer method. After overnight hybridization the blots were washed twice at low stringency (0.1% SDS, 1 × SSPE, 25 °C) and twice at high stringency (0.1% SDS, 0.1 × SSPE, 65 °C). The blots were then exposed to Kodak Biomax MR films for 1–3 days at 270 °C with intensifying screen.

Fluorescence in Situ Hybridization—Human lymphocytes were cultured in α-minimum essential medium supplemented with 10% fetal calf serum and phytohemagglutinin at 37 °C for 68–72 h. The cultures were treated with bromodeoxyuridine (0.18 mg/ml Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37 °C for 1–3 days in α-minimum essential medium with thymidine (2.5 μg/ml). Cells were harvested and fixed on slides for FISH analysis. FISH detection was as described previously (28). Essentially, slides...
were baked at 55 °C for 1 h. After RNase treatment, the slides were denatured in 70% formamide and 2 × SSC for 2 min at 70 °C followed by ethanol dehydration. Human SLAP probe was biotinylated with dATP using the Life Technologies, Inc., BioNick labeling kit and denatured at 75 °C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulfate. The slides were incubated with the probes overnight, washed, and analyzed. FISH signals and DAPI banding pattern was recorded separately by photography, and assignment of the FISH mapping data with chromosomal bands was achieved by superimposing the FISH signals with the DAPI-banded chromosomes as described previously (29).

**RESULTS**

**Sequence Analysis of SLAP Predicts an Acidic Amphipathic α-Helical Membrane Protein—**Antibodies to highly purified canine sarcolemma were raised and characterized previously (11). This antisera was used to screen a rabbit heart λ gt-11 cDNA expression library to isolate cDNAs encoding molecular components of the sarcolemma. Two immunoreactive clones (LD1 and LD2) were isolated, subcloned, and sequenced. The two independent cDNAs shared 100% identity over a 900-bp overlap (Fig. 1A). Both clones were polyadenylated but at different sites since each had poly(A) tails and putative polyadenylation signals within 20 bp of the site of polyadenylation (Fig. 1B). To obtain the full-length sequence, a 366-bp EcoRI/AccI fragment from LD1 was used to screen a randomly primed rabbit heart λ gt-11 cDNA expression library. Three overlapping clones R3a, R4b, and R4t were isolated from this screening (Fig. 1A). The resulting sequence obtained from the overlapping clones LD1, LD2, R3a, R4b, and R4t was designated to encode sarcolemmal associated protein (SLAP) (Fig. 1A).

The SLAP cDNA had a large 5'-untranslated region of 757 ns and an even larger 3'-untranslated region of 1751 nt (Fig. 1A). A large open reading frame extended from position 209 to 2675. There was a potential in-frame start codon at nucleotide 362, but it showed very poor agreement with the Kozak consensus start site (30). If this methionine was utilized, the predicted protein would be 89 kDa. The methionine codon which agreed very well with the Kozak consensus site was found at position 758, and if this was considered to be the initiating methionine the predicted polypeptide would be 639 amino acids in length and have a molecular mass of 73.7 kDa. However, to discern whether these two start sites can be effectively used an in vitro transcription/translation reaction was performed using two expression constructs. One construct utilized the methionine at 362 and the other only used the methionine at 758. Fig. 2 shows that construct using the second in-frame methionine (Kozak consensus) produced a protein migrating with apparent molecular mass of 81 kDa in SDS-PAGE. The construct containing the first ATG (non-Kozak consensus) produced a protein of 94 kDa but much less efficiently. Thus, although both ATGs can be used, the preferred initiator is likely to be at position 758 based on these data. The second ATG was considered to be the initiating methionine for the SLAP protein. The predicted protein would be acidic with a calculated pI of 4.9. The negatively charged amino acids were not evenly dispersed throughout the protein with the majority being in the carboxy-terminal 370 amino acids of the protein. A hydropathy plot (31) illustrated that the SLAP protein was highly hydrophilic except for its carboxy-terminal tail (amino acids 612–637) which several algorithms predicted could be a potential transmembrane domain (32, 33). However, the PSORT sequence analysis program predicted that SLAP would not contain a potential signal sequence (16). Analysis with a coiled-coil prediction program, COILS, revealed that large segments of SLAP protein could potentially adapt a coiled-coil structure (17). Furthermore, two regions (amino acids 465–493 and 514–535) were predicted to be potential leucine zippers, a specialized form of the coiled-coil motif where a leucine is found at every seventh amino acid (Fig. 1B) (34, 35). These two potential leucine zippers are likely part of an 11-heptad coiled-coil structure since of the 11 heptads the α positions are occupied by 8 non-polar residues (Ala, Cys, Trp, 4 Leu, and 1 Met) and 2 polar or charged residues (Ser and Arg), and at the d position 9 are non-polar residues (Leu) and 2 polar residues (Gln and Ser) (Fig. 3). A helical wheel plot of this region showed an alignment of the hydrophobic residues at positions a and d of the α helix (Fig. 3). Five potential i + 3 and i + 4 intrahelical ionic attractions were found as well in this coiled-coil region. If SLAP homodimerized in a parallel manner then there would be two attractive and two repulsive interchain ionic interaction between the i and i' + 5 positions (Fig. 3). If SLAP homodimerized in an anti-parallel fashion then there would be six repulsive and three attractive interchain ionic interactions. SLAP protein contained numerous putative phosphorylation sites for casein kinase II (20 sites), protein kinase C (12 sites), and a single putative cAMP-dependent protein kinase phosphorylation site (Fig. 1B). Two potential glycosylation sites were predicted as well (Fig. 1B). If the first non-Kozak ATG was utilized for translation initiation, SLAP would still be without a signal sequence, and the additional amino-terminal sequence would not conform to the coiled-coil structure predicted for the rest of the polypeptide. A BLAST search of GenBank revealed that SLAP shared 44% overall sequence identity with TOPAP, a topographically radiated antigen of the chick visual system (36). SLAP exhibited over 90% sequence identity at the nucleotide level with several expressed sequence tags clones from different human tissues (GenBank accession numbers N98679, N21213, AA136875, and D58115). These sequences are likely partial cDNAs of the human orthologue of SLAP. SLAP showed extended low level homology (25-30% at the amino acid level) with myosins and other coiled-coil proteins in their rod regions.

**Tissue-specific Expression of SLAP Transcripts—**To examine what tissues other than heart expressed SLAP, a 1.1-kb EcoRI/XbaI fragment from LD1 was used to probe a Northern blot

![Fig. 2. In vitro transcription/translation shows that the Kozak ATG is used efficiently. Constructs containing only the Kozak consensus start site (ATG2) or a construct containing both the Kozak and the non-Kozak consensus start site (ATG1) were in vitro transcribed and translated, and the polypeptides were separated on a 10% SDS gel. Luc, luciferase protein; H2O, water control; ATG1, construct with both the potential non-Kozak and Kozak start sites; ATG2, construct with only the Kozak consensus start site. Filled arrowhead indicates polypeptide produced by ATG1; open arrowhead indicates polypeptide produced by ATG2; arrow indicates luciferase protein, and the lines denote the mobility of the Life Technologies, Inc., prestained protein standards of molecular masses: 221, 133, 93, 67, 56, 42, 28, and 23 kDa.](504x729)
containing RNA from various rabbit tissues. In Fig. 4A a ubiquitous 5.9-kb transcript was clearly seen in brain (lane B), heart (lane H), slow twitch skeletal muscle (S), and fast twitch skeletal muscle (F). The 5.9-kb transcript was detected in kidney, spleen, and pancreas (data not shown). Two smaller transcripts of 3.5 and 4.6 kb were highly expressed in heart and slow twitch muscle but not in fast twitch muscle. Smooth muscle (aorta, stomach, uterus, and esophagus) expressed the smaller transcripts as well (data not shown). Since the two muscle-specific transcripts were significantly smaller than SLAP, different inserts from SLAP were radiolabeled and used to probe Northern blots to determine approximately where the 5' ends of the cardiac transcripts began. In panel a of Fig. 4b a 600-bp SacI/EcoRI probe from R3a hybridized only to the large transcript in fast twitch skeletal muscle (lane SK) and heart (lane H) and not at all to the smaller muscle-specific ones. However, a 366-bp AvaI/EcoRI probe from LD1, just 132 bp from the R3a probe, recognized all three transcripts in heart.

Fig. 3. Acidic amphipathic nature of the leucine zipper region of SLAP. A helical wheel plot shows the alignment of hydrophobic residues at the a and d positions of the α helix in a segment (amino acids 432–538) that covers the two leucine zippers. Seven glutamates in a row line up at the g position giving the helix an acidic face as well as the hydrophobic one given at positions a and d. Potential i + 3 and i + 4 intrahelical attractions are shown by solid arrows. Equal number of interhelical attractions (solid arrows) and interhelical repulsions (dashed arrows) would be predicted if SLAP homodimerized.
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was used. Two distinct transcripts possessing different 5′ ends from the technique of rapid amplification of cDNA ends (Fig. 4) were fractionated by formaldehyde agarose gel electrophoresis and transferred to a nylon membrane. Aliquots of total rabbit RNA (20 μg) were size-fractionated by formaldehyde agarose gel electrophoresis and transferred to a nylon membrane. α, SLAP transcripts are expressed in a varied tissues. An EcoRI/XbaI insert from LD1 was radiolabeled with [α-32P]dCTP and used to probe the blot. Multiple transcripts were seen in heart (H) and slow twitch skeletal muscle (S), but only the 5.9-kb transcript was seen in brain (B) and fast twitch skeletal muscle (F).

**FIG. 4.** Tissue-specific expression of SLAP transcripts in various rabbit tissues. Aliquots of total rabbit RNA (20 μg) were size-fractionated by formaldehyde agarose gel electrophoresis and transferred to a nylon membrane. α, SLAP transcripts are expressed in a varied tissues. An EcoRI/XbaI insert from LD1 was radiolabeled with [α-32P]dCTP and used to probe the blot. Multiple transcripts were seen in heart (H) and slow twitch skeletal muscle (S), but only the 5.9-kb transcript was seen in brain (B) and fast twitch skeletal muscle (F). b, tissue-specific expression of the alternative transcripts of SLAP. a, 600-bp SacI/EcoRI fragment from R3a was radiolabeled and used as a probe to hybridize with the blots. When this probe was used, only the 5.9-kb transcript was seen in both fast twitch skeletal muscle (SK) and heart (H). b, when a slightly more 3′ probe (366-bp AvaI/EcoRI fragment of LD1) was radiolabeled and used as a probe, three transcripts (3.5, 4.6, and 5.9 kb) were seen in heart (H), and only the single 5.9-kb transcript was seen in fast twitch skeletal muscle (SK).

(Fig. 4b, panel b). This suggested that the start of the smaller transcripts must have been near the 5′ end of the LD1 clone. To determine the exact sequence of the 5′ ends of the smaller transcripts, the technique of rapid amplification of cDNA ends was used. Two distinct transcripts possessing different 5′ ends were cloned. The isoform designated SLAP1 had a unique 5′ 39 bp and then matched SLAP from position 1558 (Fig. 5). This clone had an in-frame stop at nucleotide 18 and a potential initiating methionine at position 189 (30). The predicted SLAP1 protein would be 322 amino acids in length and have a predicted molecular mass of 37.4 kDa. The larger clone, designated SLAP2, matched SLAP from nucleotide 1386 but a region (nt 1496 to 1557) was deleted (Fig. 5). A strong potential Kozak start site was found at position 21, and the resulting protein would be 402 amino acids in length and have a predicted molecular mass of 46.3 kDa. The largest transcript would encode a protein now termed SLAP3. Both SLAP1 and SLAP2 transcripts have been expressed in mammalian cells and shown to produce polypeptides that migrate with apparent molecular mass of 35 and 45 kDa respectively.

**SLAPs Are Integral Membrane Proteins**—Since the SLAP proteins were not predicted to contain a signal sequence, their subcellular distribution and the nature of their association (peripheral or integral) with the sarcolemma needed to be examined. To study the subcellular distribution of the SLAP proteins, specific antibodies were raised to a maltose fusion protein containing the carboxyl 370 amino acids from SLAP3 that was common to all SLAPs. The antiserum was cleared of antibodies specific to the maltose binding protein and then affinity purified on a SLAP fusion protein Affi-Gel column. Fig. 6a shows that the purified antibodies recognized four polypeptides of approximately 81, 63, 45, and 35 kDa in rabbit heart fractions (lanes 2 and 3) but not in the cytosolic fractions (lane 1). The smallest protein was the most abundant in cardiac membranes consistent with the mRNA data that showed the smallest transcript was the most abundant. As well, these data were consistent with the reactivity of the anti-sarcolemmal antibodies, affinity purified from the original LD1 plaque (Fig. 6b) which shows that these antibodies recognized three polypeptides of 81, 63, and 35 kDa in rabbit heart membrane fractions (lane A) as well as porcine (lane B) and bovine heart membranes (lane C). To determine if the SLAP proteins associated with the membrane through peripheral binding or an integral membrane segment, cardiac sarcolemmal membranes were incubated on ice with high salt buffers, low pH buffers, high pH buffers, urea, or detergent and then pelleted. None of the treatments could solubilize the SLAP proteins from the membrane except for extraction with detergent (Fig. 7B, lane 7).

To investigate further the localization and membrane targeting of SLAP, P19 embryonal carcinoma cells were transfected with a SLAP expression construct in which the SLAP3 protein was tagged at amino acid 184 with the 6MYC tag epitope (23). Stably expressing clones of P19 cells were then isolated by selection with G418 (24). The majority of P19 cells were flat, and in these cells a clear immunofluorescent signal was seen at 2 J. T. Wigle, P. Wielowleiski, W. A. Staines, M. Salih, and B. S. Tuana, manuscript in preparation.
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the membrane with the anti-MYC monoclonal antibody (Fig. 8a) indicative of SLAP being targeted to the plasma membrane of the transfected P19 cells. Interestingly, the expression of SLAP was much reduced or absent at the regions of the cell membrane that were devoid of contact with neighboring cells. To determine whether the SLAP staining between adjacent cells reflects the density of SLAP protein or an increased height of the cell at the point of cell-cell contact, confocal microscopy was used (Fig. 8b, panel A). Further analysis using confocal imaging revealed that two different populations of transfected P19 cells could be discriminated on the basis of their SLAP localization. In one population of cells that appeared flat, SLAP was concentrated exclusively at the plasma membranes that were juxtaposed, whereas in a second smaller population of cells which had a rounded bipolar cell shape, SLAP was uniformly distributed in the membrane as well as an intracellular compartment (Fig. 8b, panel B). There was no immunofluorescence signal detected with the MYC monoclonal antibody in untransfected or P19 cells transfected with empty vector (data not shown). Furthermore, immunostaining with anti-SLAP antibodies also gave the same pattern of staining as seen with the MYC antibody (data not shown).

SLAPs Localize to Cardiac Membranes—To investigate the subcellular distribution of SLAP in situ, immunohistochemical localization of SLAP in cryosections of adult rat heart was performed with antibodies raised against recombinant SLAP fusion protein. SLAP staining was localized to the sarcolemma as well as a reticular pattern resembling the transverse tubules and/or sarcoplasmic reticulum (Fig. 9A). The same staining pattern was observed with three different anti-SLAP antisera including the purified antibodies. SLAP staining was abolished by preincubation with maltose binding protein-SLAP fusion protein but not by maltose binding protein alone (data not shown). SLAP staining was compared with distribution of the membrane-associated protein dystrophin. Staining of rat heart cryosections with purified anti-dystrophin antibodies showed that dystrophin localized predominantly to the sarcolemma membrane with no staining of the intracellular membranes (Fig. 9B). The intracellular staining pattern observed with anti-SLAP was similar to that seen with monoclonal antibodies to the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum implying that SLAPs may also reside in this membrane system (data not shown).

Assignment of the SLAP Gene to Human Chromosome 3p14.3–21.2—Since SLAP cDNA probes recognized multiple transcripts by Northern blot analysis, the genomic organization of the SLAP gene was investigated. Southern analysis revealed that SLAP cDNA hybridized with a simple fragment
pattern to rabbit genomic DNA upon digestion with various restriction enzymes. The more frequent cutters such as EcoRI and BamHI gave only two bands, and a rarer cutter such as SalI generated only one fragment (Fig. 10A). This relatively simple restriction map suggested that perhaps a single gene encodes the SLAPs. To determine further the localization of this gene, the human orthologue of SLAP was cloned from a human cardiac cDNA library by screening with a rabbit heart cDNA probe that encompassed the coding region common to all SLAPs. A human cDNA clone of 2.4 kb (nt 965–3412 of SLAP3) was isolated and sequenced to verify its identity as a SLAP orthologue (90% sequence identity) and was used in chromosomal mapping.

In situ hybridization of the cDNA probe to human metaphase spreads resulted in specific labeling at region p14.3–21.2 of chromosome 3 (Fig. 10B). The hybridization efficiency for this probe was approximately 91% under the conditions used, and among 100 mitotic figures examined, 91 of them showed signals on one pair of chromosomes. DAPI banding was used to identify the specific chromosomes, and the signal from the probe was assigned to the short arm of chromosome 3. The detailed position was further determined from the compilation of 10 different photographs as summarized. No additional locus was detected by FISH, and it is concluded that the SLAP gene is located on human chromosome 3 at region p14.3–21.2.

DISCUSSION

In the present study, we have assigned a gene to human chromosome 3p14.3–21.2 that encodes a coiled-coil membrane protein that we have named SLAP for sarcolemmal associated protein. The cDNA encoding SLAP was isolated by immunoscreening a rabbit heart cDNA expression library with antiserum raised to purified sarcolemma. Three cDNAs were identified in cardiac muscle that generated three different transcripts with unique 5’ and common 3’ sequences that would encode three SLAP proteins (SLAP1, SLAP2, and SLAP3). SLAPs could be polyadenylated at two different polyadenylation consensus sites. SLAP2 cDNA had a deletion of a putative exon that still maintained the reading frame. The results suggest that the SLAP transcripts can be both alternatively spliced as well as alternatively polyadenylated. The putative polypeptides encoded by these clones would have predicted molecular masses of 37 kDa (SLAP1), 46 kDa (SLAP2), and 74 kDa (SLAP3). For SLAP3 the putative initiating methionine was considered to be the one that agreed well with the Kozak consensus start site. A construct containing this start site could in vitro transcription/translation assays efficiently make a polypeptide that migrated with apparent molecular mass of 81 kDa in SDS-PAGE and agreed well with the size of laser micrograph (1-µm thick optical section) of P19 cells undergoing cleavage. SLAP appears in this case to be distributed uniformly at the plasma membrane and subadjacent intracellular compartment.
the largest SLAP protein seen in Western blots of cardiac membranes. SLAP was predicted to contain putative phosphorylation sites for several protein kinases as well as two potential glycosylation sites. Since the *in vitro* translated polypeptide migrated with the same molecular mass as the native protein and treatment of the membrane with glycosidases did not alter the relative migration of the SLAPs in SDS-PAGE (data not shown), we conclude that SLAPs are not glycoproteins.

Three different SLAP transcripts (5.9, 4.5, and 3.5 kb) were seen by Northern blot analysis in rabbit heart. Since SLAPs are encoded by a single gene, it is most likely that these transcripts are generated by alternative splicing. All three transcripts were expressed in all muscle types examined except for fast twitch muscle which only expressed the largest transcript. The 5.9-kb transcript was expressed, at varying levels, in all tissues examined including the brain. Polyclonal antibodies raised to recombinant bacterially expressed SLAP fusion protein recognized a major polypeptide of 35 kDa and three less abundant polypeptides of 81, 63, and 45 kDa in cardiac membrane but not cardiac cytosolic fractions. These data were consistent with the data obtained with the plaque-purified anti-sarcolemmal antibodies that recognized three polypeptides of 81, 63, and 35 kDa in cardiac sarcolemma from various species. The two smaller SLAP proteins (35 and 45 kDa) seen in Western blots are not likely derived by proteolysis from a larger precursor because we have cloned and expressed the two cDNAs that encode these polypeptides. Furthermore, genomic cloning and structural analysis of the *SLAP* gene indicate that several additional transcripts may also be generated by alternative splicing and/or promoter usage including a transcript that may encode...