J. Membrane Biol. 127, 35–47 (1992)

The Journal of
Membrane Biology

Mapping of the Calpain Proteolysis Products of the Junctional Foot Protein of the Skeletal Muscle Triad Junction

Neil R. Brandt†, Anthony H. Caswell†, Tara Brandt†, Keith Brew‡, and Ronald L. Mellgren§

†Department of Molecular and Cellular Pharmacology and ‡Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida, and §Department of Pharmacology, Medical College of Ohio, Toledo, Ohio

Summary. The Ca^{2+} activated neutral protease calpain II in a concentration-dependent manner sequentially degrades the junctional foot protein (JFP) of rabbit skeletal muscle triad junctions in either the triad membrane or as the pure protein. This progression is inhibited by calmodulin. Calpain initially cleaves the 565 kDa JFP monomer into peptides of 160 and 410 kDa, which is subsequently cleaved to 70 and 340 kDa. The 340 kDa peptide is finally cleaved to 140 and 200 kDa or its further products. When the JFP was labeled in the triad membrane with the hydrophobic probe 3-(trifuoromethyl) 3-(*m*)[^{125}I]iodophenyl) diazirine and then isolated and proteolysed with calpain II, the [^{125}I] was traced from the 565 kDa parent to M_r 410 kDa and then to 340 kDa, implying that these large fragments contain the majority of the transmembrane segments. A 70-kDa frament was also labeled with the hydrophobic probe, although weakly suggesting an additional transmembrane segment in the middle of the molecule. These transmembrane segments have been predicted to be in the C-terminal region of the JFP. Using an ALOM program, we also predict that transmembrane segments may exist in the 70 kDa fragment. The JFP has eight PEDST sequences; this finding together with the calmodulin inhibition of calpain imply that the JFP is a PEDST-type calpain substrate. Calpain usually cleaves such substrates at or near calmodulin binding sites. Assuming such sites for proteolysis, we propose that the fragments of the JFP correspond to the monomer sequence in the following order from the N-terminus: 160, 70, 140 and 200 kDa. For this model, new calmodulin sequences are predicted to exist near 160 and 225 kDa from the N-terminus. When the intact JFP was labeled with azidoATP, label appeared in the 160 and 140 kDa fragments, which according to the above model contain the GXGXXG sequences postulated as ATP binding sites. This transmembrane segment was predicted by the ALOM program. In addition, calpain and calpastatin activities remained associated with triad component organelles throughout their isolation. These findings and the existence of PEDST sequences suggest that the JFP is normally degraded by calpain in vivo and that degradation is regulated by calpastatin and calmodulin.

Key Words junctional foot protein · calpain · calmodulin · ryanodine receptor · PEST sequence · skeletal muscle triad junction

Introduction

Depolarization of skeletal muscle cell membranes induces release of Ca^{2+} sequestered in the sarcoplasmic reticulum (SR). The major release channel appears to be the massive structure protruding from the SR membrane known morphologically as the junctional foot protein (JFP). Those agents that activate (Ca^{2+}, adenine nucleotides, ryanodine, caffeine) and inhibit (Mg^{2+}, ruthenium red, calmodulin) Ca^{2+} release from SR vesicles correspondingly alter conductance when the isolated JFP is incorporated into planar lipid bilayers (Lai & Meissner, 1989). Recombinant DNA techniques have been employed to determine the 5037 amino acid sequence of the 563 kDa subunit of the homotetrameric JFP (Takeshima et al., 1989). The cDNA for this protein when transfected into CHO cells expresses caffeine-induced Ca^{2+} release (Penner et al., 1989). The three laboratories (Takeshima et al., 1989; Marks, Fleischer & Tempst, 1990; Zorzato et al., 1990) that have sequenced the JFP, however, are not in agreement as to the number and position of putative transmembrane segments and effector binding sites based on analysis of the amino acid sequence.

In 1983, Seiler et al. reported that the JFP in TC/triads was a "specific" substrate for calpain (Ca^{2+} activated neutral protease) and bound calmodulin. Recently Rardon et al. (1990) have shown that calpain treatment of the JFP either before or after incorporation into bilayers from SR vesicles increases the open probability of the Ca^{2+} release channel. The significance of the combined observations that the JFP both binds calmodulin and is proteolysed by calpain, however, has not been appreciated.

Wang, Villalobo and Roufogalis (1989), in com-

piling known calpain substrates, noted that most of these proteins contained both calmodulin binding sites and "PEDST" regions. These "PEDST" regions are hydrophilic segments rich in the amino acids proline (P), glutamate (E), aspartate (D), serine (S) and threonine (T) and are devoid of basic residues. They were hypothesized by Rogers, Wells and Rechsteiner (1986) to be indicators for rapid turnover in vivo and are now implicated as the domain for calpain recognition. Wang et al. (1989) also noted that occupation of the calmodulin binding site altered or inhibited calpain degradation which usually occurred at or near the calmodulin binding site.

We now report that calpin activity remains membrane-associated throughout the isolation of skeletal muscle triad junctions. The scission of the JFP by exogenous calpain is inhibited by calmodulin. All calpain fragments of the JFP remain associated as a single unit. Furthermore, using the "PEST-FIND" program of Rogers et al. (1986) we found eight positive PEDST segments in the JFP amino acid sequence. These combined observations suggest that the calpain fragments of the JFP should start and/or end in calmodulin binding regions. We followed the fate of sites labeled covalently with azido-ATP (8-azido [α-^{32}P]ATP) and the hydrophobic probe TID (3-(trifluoromethyl) 3-(*m*)[^{125}I]iodophenyl)diazine) (Brunner et al., 1985) during calpain degradation to construct a peptide map. To fit these fragments into the sequence of the JFP, we have proposed specific new calmodulin binding sites and possibly new transmembrane segments.

Materials and Methods

Triads were prepared from rabbit skeletal muscle following the protocol of Caswell, Lau and Brunschwig (1976) with the inclusion of protease inhibitors (0.1 mM PMSF, 1 μM pepstatin and 1 μM leupeptin). Pepstatin and PMSF were included in all solutions; leupeptin was removed prior to incubation with calpain. Dihydropyridine binding was assayed by filtration (Whatman GF/F filters) using 1 nM [^{3}H]PN 200-110 (72.6 Ci/mmol) as ligand. Ryanodine binding was assayed by filtration (Whatman GF/F filters) using 3.2 nM [^{3}H]ryanodine as (60 Ci/mmol) ligand (Kim et al., 1990). Assay volumes were 1 ml. For the purified JFP, filters were pretreated with polyethylenimine (Caswell et al., 1991). Protein was determined by the Coomassie blue method (Bradford, 1976) with bovine serum albumin as standard.

The JFP was purified from triads (approximately 50 mg) as described by Brandt et al. (1990). Triads were dissolved in 1 M KCl, 1.4% 3[3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)/phosphatidylcholine (PC) (from a stock 20% CHAPS/2% PC solution), 0.08 M $K_2H\ PO_4$, pH 7.0 and protease inhibitors (0.1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin) and the supernatant applied to a hydroxylapatite column (1.5 cm diameter, 15 cm length). The column was washed with 20 ml of 0.08 M $K_2H\ PO_4$, 0.5% CHAPS/PC, pH 7.3, and the JFP eluted with 0.18 M $K_2H\ PO_4$, 0.5% CHAPS/PC, pH 7.3. The hydroxylapatite eluate was then diluted twofold with 0.5% CHAPS/PC in 20 mM Tris 3(N morpholino)propanesulfonic acid (MOPS), pH 7.0, and loaded onto heparin-sepharose equilibrated with 0.1 M NaCl, 0.02 M Tris MOPS, pH 7.0, and 0.5% CHAPS/PC. The column was washed with 5 volumes of equilibrating buffer and eluted with 0.6 M NaCl, 0.02 M Tris MOPS, 0.5% CHAPS/PC. Leupeptin was eliminated from the final buffer.

Photolabeling with 12.5 μCi/ml azido ATP (8-azido [α-^{32}P] ATP, (ICN)) was carried out in a petri dish to maximize the exposure. The hydroxylapatite eluate was exposed to short (5 min) and long wavelength (10 min) UV radiation at 25 cm from the source using a Chromato-Vue Model CC-20 (Ultra-Violet Products) light box at 4°C. The photoproduct was then purified by heparin-sepharose chromatography.

Photolabeling of the JFP with the membrane probe TID (3-trifluoromethyl) 3-(*m*[^{125}I]-iodophenyl) diazirine) (Brunner et al., 1985) was carried out on intact triads. Triads in 5 ml 250 mM sucrose, 3 mM histidine and protease inhibitors were incubated for 30 min at 4°C in the dark with 125 μCi [^{125}I]TID. The solution was then transferred to a petri dish and exposed at 7 cm for 30 min at 4°C with a Model UVL-56 Black-Ray Lamp (366 nm) (UVP, San Gabriel, CA). The vesicles were then centrifuged through a 20% sucrose barrier and the JFP isolated as described above.

Calpain II (high Ca^{2+}-requiring) was isolated from rabbit white and red skeletal muscles following a modification of previously reported procedures (Mellgren et al., 1982). In brief, soluble proteins extracted from 1 kg of muscle were subjected to DEAE-sepharose chromatography, ammonium sulfate precipitation and molecular sieve chromatography as described. Active fractions were pooled, adjusted to 330 mM NaCl and then loaded onto a 50-ml phenyl-sepharose column equilibrated with 330 mM NaCl, 20 mM MOPS, pH 7.5, 1 mM EGTA, 1 mM EDTA and 1 mM dithiothreitol (DTT). The column was washed with 4 volumes of equilibration buffer and then eluted with a 100-ml linear gradient from the equilibration buffer to 4 mM EGTA, 1 mM DTT, pH 7.5. Active fractions were pooled, concentrated with a Centriprep (Amicon) and then dialyzed again in 20 mM Tris/MOPS, pH 7.5, 1 mM DTT, 1 mM EGTA, 1 mM EDTA and 50% glycerol. Routinely, the preparation was >80% pure by SDS-PAGE, and 1 μg completely destroyed the parent JFP in 100 μg triads.

Calpain activity during purification and in the localization studies was assayed with casein substrate as described by Dayton et al. (1976) or as modified by Mellgren and Lane (1988). Calpastatin was assayed as the inhibition of pure myocardial calpain activity (Mellgren & Lane, 1988). Test samples were boiled 5 min and then clarified by centrifugation for 10 min at 12,000 $\times$ *g* before addition to the exogenous calpain.

Sodium dodecylsulate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on either standard 5% Laemmli (1970) slab gels or on 3.5 to 10% gradient gels with a running pH at 9.3, and autoradiograms were developed at -70°C with Kodak X-OMAT film. Samples from calpain digestions were stopped by addition of Laemmli sample buffer containing 5 mM EGTA. In addition to the High MW Standard Set (Sigma) (myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin and carbonic anhydrase), nebulin (M_r 600K) and dynein (M_r 428K) were employed as M_r markers on gradient gels. Nebulin was detected in myofibrils prepared from rabbit psoas muscle (Solaro, Pang & Briggs, 1971), while dynein was detected in sea urchin sperm preparations obtained from Dr. M. Pratt, Department of Cell Biology and Anatomy, University of Miami.

Myocardial calpain II and the monoclonal antibody against the small subunit of bovine myocardial calpain II were prepared as

described by Mellgren and Lane (1988). The monoclonal antibody against the JFP was prepared from the line isolated by Kawamoto et al. (1986) and immobilized on CNBr-sepharose. Bovine brain calmodulin and rabbit skeletal muscle troponin C were gifts from Dr. J.D. Potter, Department of Molecular and Cellular Pharmacology, University of Miami. All other proteins and chemicals were obtained commercially.

Secondary structure predictions on the JFP sequence were carried out using the DELPHI program with three reiterations, and transmembrane regions were identified using the ALOM program using the Protein Identification Resource of the National Biomedical Research Foundation (Dayhoff, Burker & Hunt, 1983). Potential amphipathic helices were identified by a program based on the transforms of Finer-Moore and Stroud (1984) and McLachlan (1976), which predicts the periodicity of hydrophobicity for the windowed sequence. This program was designed by Mr. Jay Grobler, Department of Biochemistry and Molecular Biology, University of Miami School of Medicine. The "PEST-FIND" program was obtained from Dr. Martin Rechsteiner, University of Utah.

Results

Subcellular Localization of Calpain and Calpastatin

The small subunit of calpain (M_r 20K) and the inhibitor calpastatin were detected in skeletal muscle triad preparations by Western blotting procedures (*not shown*). To further localize these proteins, TC/triads were disrupted in a French press and the component organelles separated on an isopycnic gradient. Figure 1 (upper panel) shows the distributions for the dihydropyridine (solid line) and ryanodine (broken line) receptors, which are markers for transverse (T-) tubule and terminal cisternae (TC), respectively. These distribution patterns are essentially identical to those reported by us several times previously. Calpain was assayed as the Ca^{2+}-dependent hydrolysis of casein (lower panel, circles). The distribution of the bulk of the calpain activity matched approximately with the peak for the DHP receptor. Only a small amount appeared in the heavy TC in the position of high ryanodine binding. In addition, some calpain activity was detected at the top of the gradient. This presumably reflects the redistribution of calpain between membrane-bound and free at micromolar Ca^{2+} levels expected to be present in the gradient (*cf.* Gopalakrishna & Barksy, 1986). Essentially no proteolytic activity was found in the absence of Ca^{2+} (open triangles). Calpastatin activity (lower panel, filled squares), on the other hand, was primarily associated with those gradient fractions containing TC membranes. The presence of calpastatin at the top of the gradient and on the organelles implies that the calpain measurements underestimate the true calpain content and may account for

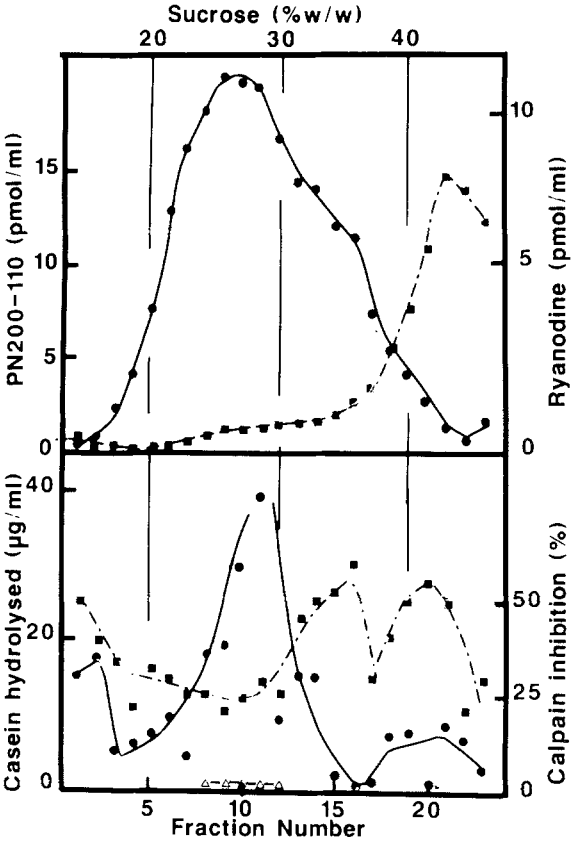

Fig. 1. Distribution of calpain and calpastatin activities among triad components. Triads, enriched by breakage and rejoining with K cacodylate (Brandt, Caswell & Brunschwig, 1980) were disrupted in the French press at 6000 psi, and the extrudate was centrifuged on isopycnic linear sucrose gradient. Dithiothreitol (5 mM) was included in all buffers to preserve calpain activity. *Upper panel:* Gradient fractions were assayed for PN 200-110 (solid line) and ryanodine (dashed line) binding as markers for T-tubule and TC, respectively. *Lower panel:* Calpain activity was assayed as the Ca^{2+}-dependent hydrolysis of casein by 10-μl aliquots over a 2 hr period at 20°C (solid line). The proteolytic activity in the absence of Ca^{2+} is shown by open triangles. Calpastatin was assayed as the inhibition of exogenous Calpain II by a 10-μl aliquot from boiled gradient fraction samples (dashed line).

the incompleteness of the correlation of calpain activity with the T-tubule marker. The reasons why calpain and calpastatin are preferentially associated with different components of the triad are at present unclear. This distribution of calpain activity was coincident with the distribution of the small subunit of calpain detected immunologically (Fig. 2). Again, the major peaks of calpain were observed with the free proteins on top of the sucrose gradient and in the region of the T-tubules. The antibody is against the 20K subunit common to both forms of calpain and thus, as with the enzyme assays, the immunoassay conditions do not distinguish between calpain

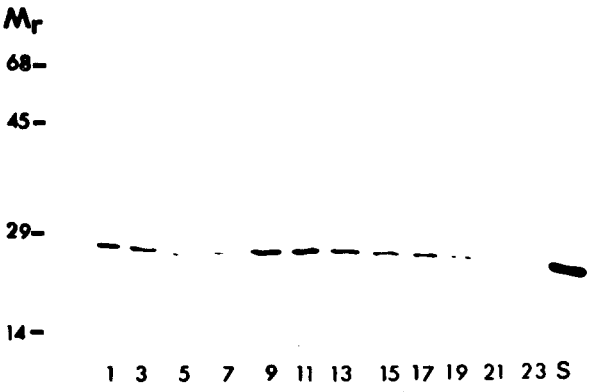

Fig. 2. Distribution of the small subunit of calpain among triad components. Aliquots (40 μl) of odd numbered fractions from the gradient in Fig. 1 were electrophoresed on a Laemmli slab gel, blocked with 2% instant milk and overlayed with a monoclonal antibody against the 20K subunit of calpain. The blots were developed with alkaline phosphatase-coupled 2° antibody and with the indolyl phosphate/nitro blue tetrazolium staining systems (Mellgren & Lane, 1988). Lane *S* was loaded with pure bovine myocardial calpain II as standard.

I (low Ca^{2+} requiring) and calpain II (high Ca^{2+} requiring).

Effect of Calmodulin on Calpain Proteolysis of JFP

Calmodulin inhibits the proteolysis of several calpain substrates (Wang et al., 1989); that calmodulin also protects the JFP from exogenous calpain II is shown in Fig. 3. Lanes *1* and *2* are the SDS-PAGE patterns of triads incubated with calpain (3.3 μg/mg triad) in the presence and absence of 100 μM calmodulin, respectively. Lanes *3* through *7* show the patterns for triads incubated with a 10-fold higher level of calpain in the presence of decreasing amounts (100 μM to 0) of calmodulin. Lane *8* shows triads incubated in a control solution without calpain. The polypeptide patterns for the triads treated with low calpain and calmodulin (lane *1*) and in the absence of protease (lane *8*) are very similar. The JFP and its endogenous breakdown product are indicated by arrow and adjacent single arrowhead, respectively. In the absence of calmodulin (lane *2*), calpain causes a decrease in the native JFP with a concomitant rise in the staining intensity of the 410K band and a band at 160K (single arrowheads). There is a barely discernible increase in a band at 340K (double arrowhead). In all of our calpain activity titrations and time course experiments, we detected a coincident increase in the staining intensity of the 410K and 160K bands preceding the increase in the 340K band. These observations suggest that the 160K and 410K are the first cleavage products of the 565K intact JFP.

The 10-fold higher level of calpain (33 μg/mg triad) in the presence of the same amount of calmodulin (100 μM) produced considerably more degradation of the 565K protein (lane *3*). In addition to the 410K and 160K pair, the band at 340K becomes prominent. Reduction of the calmodulin concentration (lanes *4–7*) caused sequential complete degradation of the 565K then 410K bands followed by loss of the 340K band. A new band (triple arrowhead) emerges at 140K, while there appears to be a loss in intensity of the 160K band. Note that a polypeptide at M_r 140K, which we have previously identified as the α subunit of phosphorylase kinase (Dombradi et al., 1984) is also susceptible to calpain hydrolysis and is protected by calmodulin. This band is distinguished from the 140K proteolytic product of JFP (triple arrowhead) by its sharpness and very slightly lower M_r. The phosphorylase kinase β subunit (128 kDa), which in our gels runs at the position of the β-galactosidase marker (116 kDa) was not proteolysed at the lower level of calpain (lane *2*), but is substantially destroyed at the higher level (lanes *3–7*). This proteolysis of the membrane-associated form of phosphorylase kinase is consistent with the well-established action of calpain or calcium-activating factor (CAF) on the soluble enzyme (Meyer, Fischer & Krebs, 1964). The fate of smaller polypeptides (<100 kDa) formed by JFP degradation cannot be tracked with intact triads. Similar progression of production of proteolytic bands was observed when calpain was held constant and the time of digestion varied.

Nebulin (600 kDa), dynein (428 kDa), myosin (205 kDa), β-galactosidase (116 kDa) and phosphorylase *b* (97 kDa) were used as standards to construct the calibration curve shown in Fig. 3 (lower panel) from gradient gels. The intact JFP monomer migrated at 500K (arrow) while the breakdown product migrated at 410K (single arrowhead) The parent JFP will be designated 565K in accord with its true molecular weight. The high M_r fragments at 410K and 340K (double arrowhead) correspond to the 350 and 315 kDa calpain products reported by Rardon et al. (1990) who used lower M_r standards. The early appearing 160K and late appearing 140K fragments are indicated by the single and triple arrowheads, respectively.

Calpain degradation of the JFP was also inhibited by troponin C as predicted by its relationship to calmodulin. On the other hand, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and aldolase, which also bind to the JFP (Kawamoto et al., 1986) did not prevent formation of lower M_r forms.

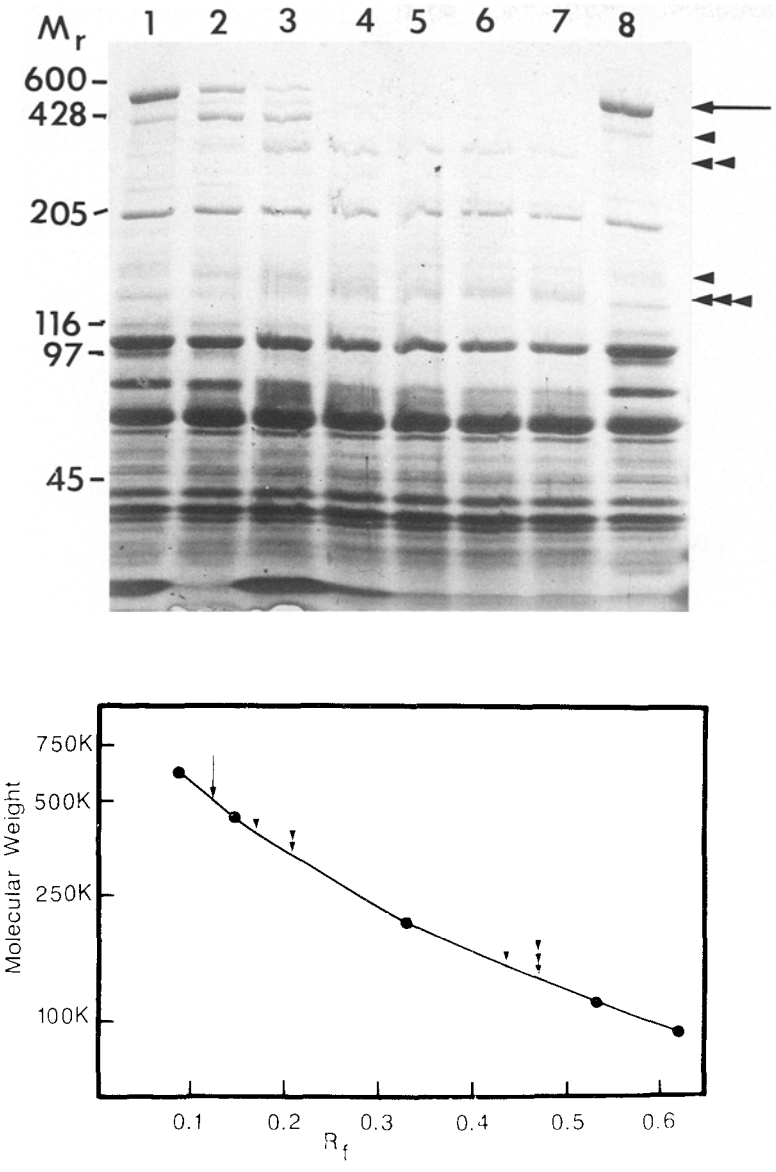

Fig. 3. Proteolysis of skeletal muscle triads by calpain in the presence and absence of calmodulin. *Upper panel:* SDS PAGE pattern for triads incubated 30 min at 20°C with 3.3 μg calpain/mg triad, lanes *1* and *2*; 33 μg calpain/mg triad, lanes *3–7*; or no calpain, lane *8*. Calmodulin was present at 100 μM, lanes *1* and *3*; 10 μM, lane *4*; 1.0 μM, lane *5*; 0.1 μM, lane *6* or absent, lanes *2*, *7* and *8*. Gel conditions: 3.5–10% acrylamide, pH 9.3 running buffer, 50 μg triad protein/lane. *Lower panel:* Calibration curve of log molecular weight *vs.* R_f using nebulin (600 kDa), dynein (425 kDa), myosin (205 kDa), β-galactosidase (116 kDa) and phosphorylase *b* (97 kDa) as standards. The JFP is marked by arrow; M_r 410K and 160K by single arrowhead; M_r 340K by double arrowhead and M_r 140K by triple arrowhead in both *upper* and *lower* panels.

Calpain Degradation of the Isolated JFP

To confirm the identity of the high M_r JFP fragments seen after calpain action on triads, we examined the degradation pattern produced by calpain treatment of the purified JFP (Fig. 4). The weight ratio of calpain to the JFP is indicated below each pair of lanes. To distinguish the polypeptides produced by JFP hydrolysis from the added calpain, part of the reaction mixture was precipitated with a monoclonal antibody against the JFP covalently linked to sepharose (lanes marked *ab*). In the absence of calpain, the bulk of the Coomasie blue staining material was associated with the parent JFP; the additional bands seen in the antibody retentate are the antibody heavy and light chains (arrowheads) and an M_r 45K contaminant apparently coming from the antibody column. At 0.02 calpain/JFP, the loss of the 565K band and an accompanying increase in the 410K and 160K are seen. All three bands were retained by the antibody.

With the increase in calpain/JFP to 0.07 and 0.2, a new band appears at 340K. This becomes the major high M_r band at the highest calpain/JFP and is bound by the antibody. If the 340K band were derived from the 410K fragment, then there should also appear a band near 70K, but the large subunit of calpain may be masking it (asterisk). A band at this M_r from the antibody column may represent the 70 kDa subunit, but we cannot rule out the possibility that the column retains some calpain that is bound to its substrate.

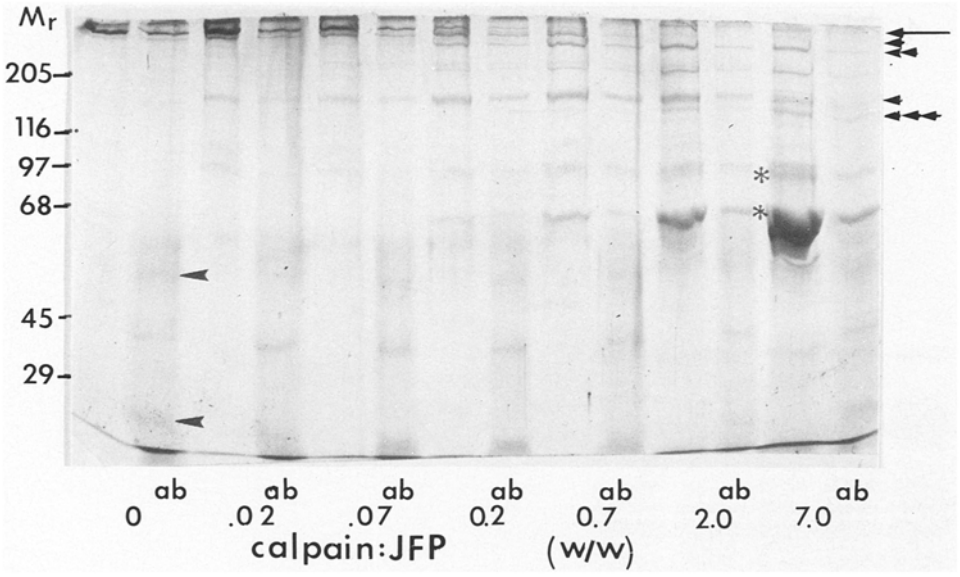

Fig. 4. Calapin proteolysis of the purified JFP. The JFP, purified as described in Materials and Methods, was incubated at 70 μg/ml in 450 μl reaction volume containing 3 mM DTT, 3 mM $CaCl_2$ and the calpain to JFP ratio indicated below each pair of lanes. After 30 min at 20°C, 60 μl were removed for ryanodine binding, 60 μl mixed with Laemmli solubilizing buffer and the remainder incubated with 50 μl mAb anti-JFP Sepharose. The mAb Sepharose, after 1 hr, was washed with 0.6 M NaCl, 20 mM MOPS, pH 7.0, four times by centrifugation and then boiled with 100 μl Laemmli solubilizing buffer. The free solution (100 μl) was then loaded onto the 5% Laemmli gel lanes marked *ab*. The light and heavy chains of the mAb are indicated by arrowheads. The large subunit of calpain and the contaminant of the calpain preparation are indicated by asterisks. The arrow and arrowheads to the right of the gel indicate the position of the parent JFP and its fragments as described for Fig. 3.

The production of a M_r 140K band is first apparent at 0.7 calpain/JFP and its intensity increases with increasing levels of calpain. The production of this band is accompanied by an increase in the appearance of a band at 200K. Both bands appear after the formation of the 340K fragment and both are retained by the antibody. The relative intensities of the 340K, 200K and 140K bands at 0.7, 2.0 and 7.0 calpain/JFP suggest that the smaller bands are being generated at the expense of the 340K band.

The antibody used to precipitate the JFP is monoclonal, and it retained all identifiable fragments of JFP even when the parent molecule was completely proteolysed. It is most likely that there is a single epitope for this antibody in the JFP sequence and therefore the antibody should react directly with only one fragment. These fragments of the JFP also eluted as a single unit from molecular sieve and protamine affinity columns (*data not shown*). We also determined that ryanodine binding assayed after proteolysis remained constant for all the samples shown in Fig. 4. These observations suggest that the fragments of the JFP remain noncovalently associated even after multiple cleavage of the primary structure and that the structure of the protein may be largely unaltered.

Identification of ATP Binding Fragments of the JFP

Ryanodine binding and Ca^{2+} conductance by the JFP are enhanced by adenine nucleotides, but the number and position of ATP binding sites are controversial. We screened for ATP binding sites on the calpain fragments by photolabeling the JFP after solubilization and hydroxylapatite chromatography with 8-azido ATP. The JFP was then purified by heparin-Sepharose chromatography. ATP labeling did not occur in the presence of 1 mM $Mg Cl_2$ and was diminished by excess unlabeled ATP. Figure 5 shows the Coomassie blue pattern (left panel) and autoradiogram (right panel) for the JFP treated with the proportion of calpain at the ratios indicated under each lane. The changes in the protein staining pattern with increasing calpain levels are similar to those in Fig. 4. For the untreated JFP, the bulk of the protein appears at 565K with detectable amounts of the 410K and 160K fragments. A contaminant in the 100K region is also present. The 410K and 160K bands are enhanced after treatment with calpain (0.001 calpain/JFP) and increase again in intensity at 0.01 calpain/JFP. At 0.1 calpain/JFP ratio, the 410K is diminished while bands at 340K and 140K become prominent. At this level of protease the par-

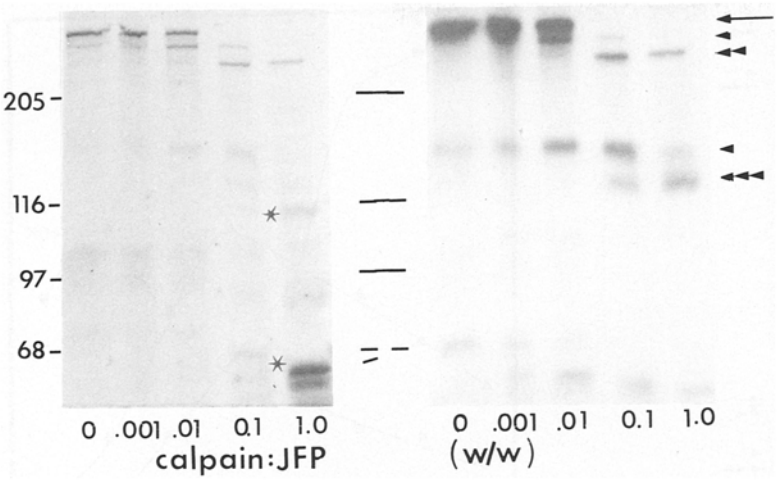

Fig. 5. ATP-binding calpain peptides of the JFP. The JFP was photolabeled with 8-azido ATP after hydroxylapatite chromatography as described in Materials and Methods and then purified by heparin chromatography. The purified protein (0.26 mg/ml) was incubated in 100 μl reaction volumes containing 3 mM $CaCl_2$, 3 mM DTT and equal volumes of JFP and calpain to give the ratios indicated under each lane. Proteolysis was carried out for 30 min at 20°C. The 5% slab gel was run until the 45K marker was eluted to maximize the separation among the high M_r fragments. Peptides coming from the calpain preparation are indicated by asterisks. The arrow and arrowheads to the right of the gel indicate the position of the parent JFP and its fragments as described for Fig. 3.

ent JFP has been almost completely destroyed. At the maximum level of calpain (1.0), the 340K and 140K bands persist while the 160K band is diminished and the 410K band is almost absent. Unlike the data shown in Fig. 4, very little product of 200K was discerned. It appears likely that the 200K fragment can be hydrolyzed so rapidly that it is not detectable. Note that the 100K band is not affected by calpain.

The autoradiogram (right panel) shows that the bulk of the ATP was incorporated into the parent JFP with some labeling of the 410K and 160K fragments. These fragments become more prominent after treatment with a low level of calpain (0.001 and 0.01). The 410K band is diminished at 0.1 calpain/JFP and absent at 1.0 calpain/JFP while the 160K is reduced only at the highest level of protease. The 340K and 140K bands first appear at 0.01 calpain/JFP. The 340K peaks at 0.1 calpain/JFP and then diminishes at 1.0 calpain/JFP while the 140K band appears to increase at the highest protease level.

Radioactivity was detected in the untreated JFP at 70–75K, which decreases with calpain treatment. There was an increase in a band running ahead of calpain with protease treatment which may be at the expense of this band. There was no indication, however, of a radioactive band at or above calpain appearing at the same concentrations as the 340K fragment of the JFP.

Calpain Fragmentation of the JFP Labeled with the Hydrophobic Probe [^{125}I]TID

Trials were incubated with the hydrophobic probe [^{125}I]TID to allow partitioning of the ligand into the membrane bilayer. Upon photoactivation, the generated carbene radical reacts primarily with lipids and those proteins surrounded by lipids (Brunner et al., 1985), i.e., transmembrane domains. In Fig. 6, upper panel, the single lane marked *TR* shows the Coomassie blue stained gel for the triads. Immediately below is the autoradiogram. The TID label is found in the JFP, the SR Ca^{2+} pump and at the dye front where membrane lipids are known to run. No other activity was apparent, indicating the specificity of the label for intrinsic membrane proteins.

The right-hand side of Fig. 6 shows the Coomassie blue pattern (upper panel) and autoradiogram (lower panel) for the JFP isolated from the TID-labeled triads after incubation with the level of calpain (wt/wt) indicated below each lane. The protein staining pattern is similar to that in the last two figures. For the untreated JFP the bulk of the protein appears as the 565K parent molecule, but the 410K and 160K fragments are also present. Between 0.03 and 0.1 calpain/JFP, a decrease in the 565K content with an increase in the 410K and 160K bands is discernible. The parent JFP is almost completely destroyed at 0.3 calpain/JFP; the 410K and 160K bands are unaltered but the bands at 340K and 140K appear enhanced. The intensity of the 200K band also appears to increase in parallel with the 140K band but is considerably lower in content. The new band at 70K is calpain (asterisk). At 1.0 calpain/JFP, the 410K is reduced while the 340K and 140K have increased. In addition to the putative contaminant at 100K, a faint band can be seen just above the dye front (~25K). This small peptide increases in intensity with increasing levels of calpain.

The lower panel shows that the bulk of the incorporated TID is in the parent 565K band for the untreated JFP with some labeling of the 410K fragment. In parallel with the changes in the Coomassie blue pattern, the TID label sequentially appears in the 410K and 340K bands. Thus the 340K band most

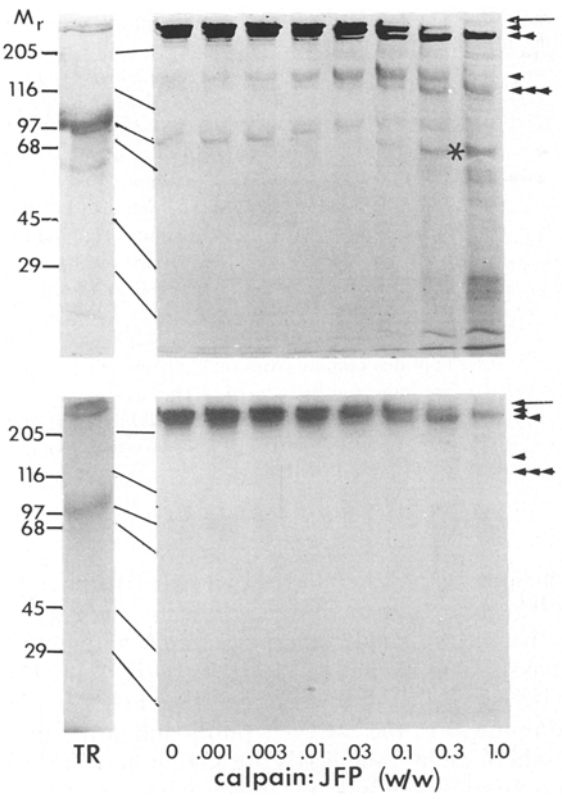

Fig. 6. Calpain fragments of the JFP labeled with [^{125}I]TID. Triads were equilibrated and photoreacted with the hydrophobic probe [^{125}I]TID. Lanes marked *TR* show the Coomassie blue staining pattern (*upper*) and autoradiogram (*lower*) for 30 μg triads. The JFP was purified as described in Materials and Methods and incubated at 40 μg/100 μl in heparin elution buffer containing 3 mM DTT, 3 mM $CaCl_2$ and calpain at the ratios indicated below each lane for 30 min at 20°C. Reaction mixtures were electrophoresed on a 5% Laemmli slab gel. *Upper panel:* Coomassie blue stained gel; *lower panel:* autoradiogram. Large subunit of calpain is indicated by asterisk. The arrow and arrowheads to the right of the gel indicate the position of the parent JFP and its fragments as described for Fig. 3.

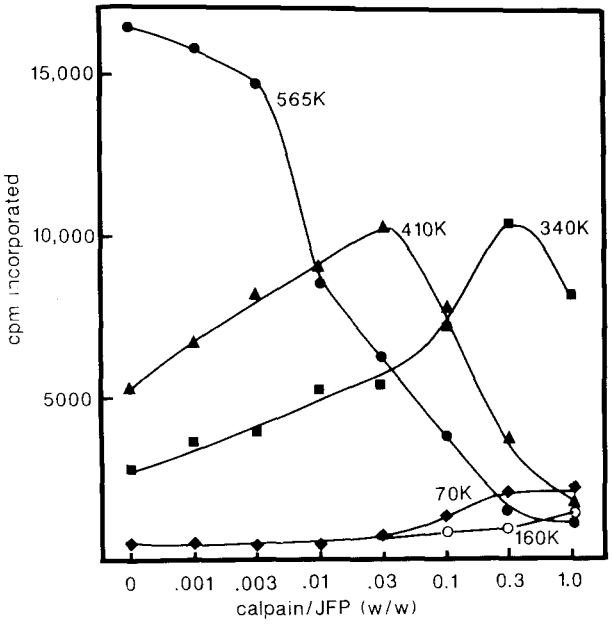

Fig. 7. Radioactivity incorporated in calpain fragments of the JFP by photo-reaction with [^{125}I]TID. Slices of the SDS-PAGE gel shown in Fig. 6 corresponding to the calpain fragments were cut out and counted using a γ counter. Bands were identified from either the Coomassie blue staining or the autoradiogram. Solid circles, 565K JFP parent; triangles, 410K fragment; squares, 340K fragment; diamonds, 70K fragment; open circles, 160K fragment.

likely contains the bulk of the hydrophobic or transmembrane segments. The autoradiogram shows no clearly discernible labeling in the 160K. A slight degree of labeling of the 200K band is observed which represents a significant specific activity since the band is faint in the stained gel. A faint degree of labeling, however, can be seen immediately above the calpain band at the highest calpain levels. The labeling of the 100K band remains constant while the 25K band increases with increasing calpain.

Because of the faint development of the bands below 340K, we cut out and counted the radioactivity in all bands (565K, 410K, 340K, 160K, 140K, 70K and 25K) that may have been of JFP origin. The total activity incorporated into these bands remained constant up to 0.1 calpain/JFP and deceased only by 25% at 1.0 calpain/JFP. Figure 7 shows that the vast bulk of the counts first appears in the parent JFP then the 410K and finally in the 340K. In contrast, the 160K band remained very low and constant despite its enhancement as a stained band. The radioactivity in the 200K, 140K and 25K bands increased only at 1.0 calpain/JFP; however, ^{125}I at a band immediately above the calpain subunit increased at 0.3 calpain/JFP and its development pattern paralleled the development of the 340K fragment.

Discussion

Unlike other proteases, calpain appears to cleave the JFP in an ordered sequence of susceptible sites; that is, the first site must be cleaved before the second site becomes exposed. Our data show that the parent JFP (565K) is first cut into fragments of 410K and 160K. The latter appears to be stable, whereas the 410K is cut to 340K. The 70K companion piece to the 340K was not adequately detected in these experiments, in part because it may be obscured by the large subunit of calpain itself when the proteoly-

sis products are detected by protein staining. This may even be the case when the reaction products were extracted with the monoclonal antibody against the JFP since it was possible that calpain was tightly attached to the JFP.

The 340K fragment is subject to cleavage at higher calpain concentrations. One potential product has M_r 200K. However, this fragment is formed at variable concentrations. It is possible that it is further cleaved to smaller products. A fragment at 140K could be produced concomittant with the 200K on breakdown of the 340K polypeptide. An alternative source of the 140K fragment is through cleavage of the 160K fragment. Two arguments may be made against this. (i) In the TID experiments the 160K fragment was unlabeled while the 140K was labeled, albeit weakly. Radioactivity counts in the 160K fragment at its peak protein staining concentration was 290 counts per min (cpm) above control while in the 140K fragment at peak protein concentration were 1250 cpm above control. (ii) Perusal of the sequence suggests no likely points of proteolysis. As will be discussed later, the action of calpain requires a calmodulin binding domain close to the proteolysis site. No amphipathic basic domains are present at the required cleavage sites (200 amino acids from either end) within the 160 kDa fragment.

The progression of peptides (565K to 410K to 340K to 140K) was seen in time course experiments (not shown here), calpain concentration dependence (Figs. 4–6) and in calmodulin inhibition experiments (Fig. 3) with the JFP in the intact triad, and in the isolated state. These observations suggest that all susceptible sites are present on the cytoplasmic surface of the SR. Furthermore, all of the fragments generated by calpain appear to remain as an intact unit in molecular sieve and affinity chromatography. These observations imply that the fragments of the JFP are held together by intra or intermolecular disulfide bridges or noncovalent bonds. That the quarternary structure is maintained even after extensive calpain proteolysis is indicated by the observation that ryanodine binding is not decreased. Ryanodine binding is postulated to be a property of the tetrameric JFP molecule (Lai et al., 1989).

The [^{125}I]TID experiment determines the position of the fragments within the intact molecule. The TID was photoreacted in the intact vesicle in order to maximize the labeling of transmembrane segments since the ligand partitions freely into the hydrophobic portion of the membrane. The protein sequence suggests that almost all of the transmembrane domains are restricted to the last 20% of the molecule. Since the 410K and 340K fragments are heavily labeled with TID, these peptides must contain the C-terminus. The 160K peptide was not labeled; however, the 70K peptide from the 410K fragment contained some label, and the content of label increased with the formation of the companion 340K fragment. The TID label could not be traced unambiguously beyond this point. The sum of the newly formed 140K and 200K fragments at the maximum calpain/JFP accounted for less than 25% of the loss of radioactivity in the 340K peptide. The content of 200K fragment in the labeling experiment was low but a distinct band was clearly visible in the autoradiogram, suggesting that the specific activity is very high and therefore that the 200 kDa fragment is C terminal.

In general, calpain cuts its substrates at or near calmodulin binding domains (Wang et al., 1989). Here we have shown that calmodulin inhibits calpain degradation of the JFP in a concentration-dependent manner (Fig. 3) and that protection is in the reverse order of the generation of high M_r peptides. The TID data indicate cut sites at 160K and at 220K (560K less 340K) from the N-terminus. Calmodulin sites have not been previously predicted at those sites. We scanned the JFP sequence with the DELPHI secondary structure prediction program and with a program which through a Fourier transform predicts the periodicity of hydrophobicity of the windowed sequence. The first sequence listed in Table A at 156K is a positively charged amphipathic helix while the second sequence at 223K is positively charged and amphipathic with an adjacent helix nucleation region. Considering that the 140K peptide generated from the 340K fragment contained little TID label, we suggest that it may be generated from the N-terminal portion, i.e., starting from amino acid ~2000. That would put a third cleavage site at 365K. The calmodulin site predicted by Marks et al. (1990) at 377K (amino acids 3358–3374) is listed third in the Table. Although the alternative cleavage point at 425K is near the 406K (amino acids 3614–3637) calmodulin site predicted by Takeshima et al. (1989), other reasons to be presented suggest that this is a less likely cut point. Our calpain data do not rule out the existence of other calmodulin sites which are not attacked by calpain.

The fact that calmodulin inhibited the action of calpain suggested that the JFP might be a "PEST-type" calpain substrate. PEDST sequences are hypothesized to be the site of binding for calpain and are normally linearly near the susceptible calmodulin binding sites (Wang et al., 1989). We scanned the published amino acid sequence of the JFP with the "PEST-FIND" program provided by Dr. Martin Rechsteiner (Rogers et al., 1986). The program found the eight sequences with positive "PEST" scores listed in Table B as well as several weak (PEST score <0) sequences. The high number of

Table. Domains of the JFP

A. Calmodulin binding domains		M_r
Amino acids	Sequence	
1383–1400	KNKKRGFLFKAKKAAMMT	156K
1974–1996	RSRYALLMRAFTMSAAETARRTR	223K
3358–3374	FIPTIGRLRKRAGKVVA	377K

B. PEDST sequences		PEST score	M_r
Amino acids	Sequence		
1302–1324	RCTAGATPLAPPGLQPPAEDEAR	0.267	147K
1354–1372	KEGTPGGTPQPGVEAQPVR	0.686	153K
1864–1895	KMIEPEVFTEEEEEEEEEEEEEEEEEE DEEEK	33.1	211K
2041–2064	HCGIQLEGEEEEPEEETSLSSR	13.7	230K
3287–3311	RGPEAPPPALPAGAPPPCTAVTSDH	4.81	370K
4230–4295	KMELFVSFCEDTIFEMQIAAQISEPE GEPEADEDEGMGEAAAEGAEEGAAGA EGAAGTVAAGATAR	1.08	478K
4446–4474	RPEGAGGLGDMGDTTPAEPPTPEGS PILK	8.08	500K
4581–4650	KVSDSPPGEDDMEGSAAGDLAGAGS GGGSGWGSGAGEEAEGDEDENMVYY FLEESTGYMEPALWCLSLLH	2.51	514K

C. Transmembrane segments		P:I	M_r
Amino acids	Sequence		
599–614	VLDVLCSLCVCNGVAV	0.24	67K
1514–1530	LVIGCLVDLATGLMTFT	0.13	170K
1839–1855	VPVLKLVSTLLVMGIFG	5.8×10^{-3}	207K
2555–2570	CLAVLPLITKCAPLFA	0.037	287K
4024–4041	VVMLLSLLEGNVVNGMIA	0.50	452K
4331–4346	LAALLWAVVARAGAAG	0.56	486K
4564–4583	FLALFLAFAINFILLFYKVS	2×10^{-4}	513K
4644–4660	WCLSLLHTLVAFLCIIG	9×10^{-4}	522K
4837–4855	LVMTVGLLAVVVYLYTVVA	9×10^{-4}	544K
4921–4937	FFFVIVILLAIIQGLII	9×10^{-7}	554K

Calmodulin sequences were determined by the combined output of the DELPHI program for secondary structure and a program based on the Fourier transforms of Finer-Moore and Stroud, which predicts the periodicity of hydrophobicity in the windowed sequence. The latter program was developed by Mr. Jay Grobler. PEDST sequences were determined by the PEST-FIND program from Dr. Martin Rechsteiner. Transmembrane segments were determined by the ALOM program using the Protein Identification Resource of the National Biomedical Research Foundation. Sequences given are the predicted minimum core of the transmembrane segment and may be extended two or three amino acids to either end.

PEDST sequences is unusual even for the large length of the JFP monomer; most proteins including fodrin (230 kDa) have only one or at most two domains (*cf.* Wang et al., 1989). Seven of the JFP sequences are in the range found for other calpain substrates; the third segment has the abnormally high score of 33, but consists almost entirely of polyglutamate rather than a more balanced content of P, E, D, S, and T found in most PEDST regions. The eighth region corresponds to an SR luminal segment predicted from the sequencing data (Takeshima et al., 1989; Marks et al., 1990; Zorzato et al., 1990). PEDST sequences are linearly near our putative calmodulin domains at 155K and 223K and near the predicted domain at 377K but not near the alternative site at 425K. Two PEDST sequences flank the

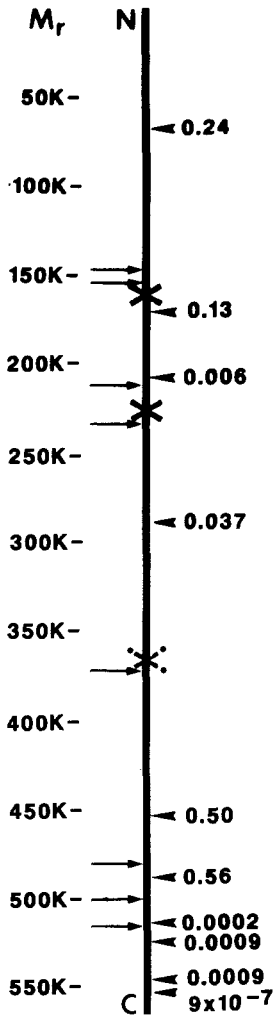

Fig. 8. Linear model for calpain scission points, PEDST regions and potential transmembrane segments. Probable calpain scission points are indicated by crosses on the sequence line; preferred alternative scission point for the 340K fragment is indicated by broken crosses. PEDST sequences as listed in the Table are indicated by arrows on the left side. Transmembrane segments as predicted by the ALOM program are indicated by arrowheads. Peripheral: integral scores indicated by right of arrowhead. *N* and *C* indicate NH_3^+ and CO_2^- terminals of the JFP.

predicted calmodulin site at 483K (Takeshima et al., 1989). We have not detected fragments that could arise from calpain proteolysis at this site. It is possible that the PEDST sequences or the 483K calmodulin binding domain are occluded or protected in the intact and partially proteolyzed protein.

From the data described above we have constructed the linear model for the JFP shown in Fig. 8. The position of the PEDST sequences are shown by arrows on the left side and the probable positions of the calpain cuts are shown by the crosses on the line. We then tested whether the predicted transmembrane segments would be consistent with our observations. The consensus segments at 512K, 521K, 543K and 552K first proposed by Takeshima et al. (1989) are assumed correct in our contention that the 410K and 340K fragments contain the C-terminus. Zorzato et al. (1990) in addition to the consensus segments predicted an additional eight transmembrane helices: six appearing in the C-terminus at 447K, 451K, 480K, 487K, 538K and 548K and a loop (designated M′-M″) at 353K and 358K. We scanned the JFP sequence using the ALOM program which predicted the 10 segments listed in Table C. The position and the probability (peripheral : integral) of these segments is shown on the right side of Fig. 8. The ALOM program indicated the consensus segments as being of high probability but only two of the additional C-terminus segments proposed by Zorzato et al. (1990) and those were of low probability (P : I = 0.5). The M′-M″ loop was not recognized. Four additional transmembrane segments in the N-terminal half, however, were indicated by the ALOM program. All of these had a higher probability than the Zorzato et al. (1990) segments and that of the segment at 207K was in the range of the consensus segments. The 207K transmembrane segment could account for the labeling of the 70K fragment. The direction of that segment must be luminal to cytoplasmic because the PEDST and putative calmodulin domains following in the sequence must be exposed to calpain in the intact triad. To be part of a transmembrane loop, the paired cytoplasmic to luminal segment must then be between the calmodulin site at 160K and the 207K transmembrane segment, suggesting the segment at 170K as a possible transmembrane segment.

The simplest model requires calpain cuts at 160K, 230K and 370K or 430K from the N-terminus of the 565K JFP with one transmembrane loop between the 160K and 230K cuts and at least two loops in the C-terminus. This model leaves out the potential transmembrane sequences at 67K and 287K since they cannot be appropriately paired into transmembrane loops that would leave the calpain cut sites exposed. The possibility that the 67K is an unpaired segment going from SR lumen to cytoplasm can be ruled out since the N-terminus of the JFP does not contain a signal sequence. We tested the ALOM program against the reported structure of the SR Ca^{2+}-ATPase (Clarke et al., 1989): 8 of the 10 reported sequences, but no unreported sequences were predicted. This suggests that we may have missed transmembrane segments. The minimal requirements of a model for our data, however, are only two transmembrane segments (one loop) in the 70K fragment and the four consensus transmembrane segments (two loops) in the C-terminus of the JFP.

The ATP-labeling data serve as one test for our proposed positioning of calpain cut sites (160K, 230K and 370 or 430K from the N-terminus) of the JFP. Although the protein was heavily labeled by azido ATP, no consensus sequences of the GKT motif (A/G XXXX GK T/S) (Walker et al., 1982) exists except for the weak homology at amino acids 417–424 (GLDSFSGK). On the other hand, Takeshima et al. (1989) stated that there existed several consensus sequences of the motif for nucleotide binding GXGXXG in the JFP but cited only the one at amino acids 4449–4457 in the C-terminus. Our binding data showed that both the 160K and 410K fragments were labeled. The GXGXXG motif is present at amino acids 699–706 and 1195–1200, both within the 160K; the latter, however, has been questioned by Zorzato et al. (1990) on the grounds of secondary structure. After the next cleavage, the 340K fragment is labeled while the 70K fragment is not. The 340K peptide contains binding motifs at 2370–2375 and 4602–4607 in addition to the one cited by Takeshima et al. (1989). The one at 4602–4607 is most likely to be luminal. Our ATP data indicated that the 140K fragment of the 340K was heavily labeled. Radioactivity was detected in the 200K region only upon prolonged exposure, along with the appearance of several lower M_r bands. If the 140K fragment is formed from the N terminus of the 340K peptide, i.e., 225K to 365K in the intact protein, then the most probable ATP binding site is at amino acids 2370–2375.

The fact that the JFP appears to be a PEDST-type calpain substrate implies that calpain is responsible for the normal turnover of the JFP in vivo. Consistent with this implication, we found calpain activity and calpastatin associated with triads isolated from skeletal muscle. Our enzymatic and immunological assays did not distinguish between the low (μM) Ca^{2+} (calpain I) and high (mM) Ca^{2+} (calpain II) requiring forms of the protease. The association of both forms of calpain and calpastatin with skeletal muscle membranes, however, has been previously reported (Gopalakrishna & Barsky, 1986). Immunoelectron-microscopic studies have shown calpain I activity to be located at or near the T-tubule in rat skeletal muscle (Yoshimura et al., 1986). The small amount of calpain associated with the heavy TC may be significant if calpain can bind tightly to the JFP as suggested in Fig. 4. Rogers et al. (1986) have correlated PEDST sequences with rapid turnover in vivo. For the JFP in normal tissue, the turnover is most likely controlled by calpastatin and calmodulin. Rardon et al. (1990) have projected that calpain destruction of the JFP is associated with pathological states of muscle, and we have previously reported that calpain can break isolated triads (Kim et al., 1990).

The observation that calpain activity is retained by the triad throughout its isolation may be of importance for the intrepretation of in vitro experiments. The level of Ca^{2+} required to fuse triad vesicles to lipid bilayers may be sufficient to activate associated calpain and thereby alter the gating properties of the incorporated channel. Similarly, any test for contractility at pCa 4 in digitonin, α-toxin or mechanically skinned muscle fibers may be associated with activation of membrane-bound calpain, which could alter the Ca^{2+} sequestration and release properties of the SR (*cf.* Kasuga & Umazume, 1990). It is noteworthy that the calpain proteolyzed JFP loses its capacity to bind aldolase (Brandt et al., 1990), which may act as a sink for inositol 1,4,5-trisphosphate in vivo (Thieleczek, Mayr & Brandt, 1989).

We wish to thank Mr. Jay Grobler for the use of his secondary structure program and Prof. Martin Rechsteiner for the "PEST-FIND" program. Dr. William Strauss performed the initial "PLOT-structure" program. The secretarial skills of Ms. Norma Jacobs are gratefully acknowledged. This work was supported by NIH grants AR 21601, GM 21363 and HL 36573, by the Muscular Dystrophy Association of America, and by a Grant-in-Aid from The American Heart Association, Florida Affiliate.

References

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding. *Anal. Biochem.* **72:**248–254

Brandt, N.R., Caswell, A.H., Brunschwig, J.-P. 1980. ATP-energized Ca^{2+} pump in isolated transverse tubules of skeletal muscle. *J. Biol. Chem.* **225:**6290–6298

Brandt, N.R., Caswell, A.H., Wen, S.-R., Talvenheimo, J.A. 1990. Molecular interactions of the junctional foot protein and dihydropyridine receptor in skeletal muscle triads. *J. Membrane Biol.* **113:**237–251

Brunner, J., Franzusoff, A.T., Luscher, B., Zugliani, C., Semenzo, G. 1985. Membrane protein topology: Amino acid residues in a putative transmembrane of helix of bacteriorhodopsin labeled with the hydrophobic carbene-generating reagent 3-(trifluoromethyl)-3-*m*-([^{125}I] iodophenyl) diazirine. *Biochemistry* **24:**5422–5430

Caswell, A.H., Brandt, N.R., Brunschwig, J.-P., Purkerson, S. 1991. Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95000 protein (Triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles. *Biochemistry* **30:**7507–7513

Caswell, A.H., Lau, Y.H., Brunschwig, J.-P. 1976. Ouabain-binding vesicles from skeletal muscle. *Arch. Biochem. Biophys.* **176:**417–430

Clarke, D.M., Loo, T.W., Inesi, G., MacLennan, D.H. 1989. Localization of high affinity Ca^{2+} binding sites within the predicted transmembrane domain of the sacroplasmic reticulum Ca^{2+} ATPase. *Nature* **339:**476–478

Dayhoff, M.O., Burker, W.C., Hunt, L.T. 1983. Establishing homologies in protein sequences. *Methods Enzymol.* **91:**524–545

Dayton, W.R., Reville, W.J., Goll, D.E., Stromer, M.A. 1976. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. *Biochemistry* **15:**2159–2167

Dombradi, W.K., Silberman, S.R., Lee, E.Y.C., Caswell, A.H., Brandt, N.R. 1984. The association of phosphorylase kinase with rabbit muscle T-tubules. *Arch. Biochem. Biophys.* **230:**615–630

Finer-Moore, J., Stroud, R. 1984. Amphipathic analysis and possible formation of the ion channel in an acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **81:**155–159

Gopalakrishna, R., Barsky, S.H. 1986. Hydrophobic association of calpains with subcellular organelles compartmentalization of calpains and the endogenous inhibitor calpastatin in tissues. *J. Biol. Chem.* **261:**13936–13942

Kasuga, N., Umazume, Y. 1990. Deterioration introduced by physiological concentration of calcium ions in skinned muscle fibers. *J. Muscle Res. Cell Motil.* **11:**41–47

Kawamoto, R.M., Brunschwig, J.-P., Kim, K.C., Caswell, A.H. 1986. Isolation, characterization, and location of the spanning protein from skeletal muscle triads. *J. Cell. Biol.* **103:**1405–1414

Kim, K.C., Caswell, A.H., Brunschwig, J.-P., Brandt, N.R. 1990. Identification of new subpopulation of triad junctions isolated from skeletal muscle. Morphological correlations with intact muscle. *J. Membrane Biol.* **113:**221–235

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277:**680–685

Lai, F.A., Meissner, G. 1989. The muscle ryanodine receptor and its instrinsic Ca^{2+} channel activity. *J. Bioenerg. Biomembr.* **21:**227–246

Lai, F.A., Misra, M., Xu, L., Smith, H.A., Meissner, G. 1989. The ryanodine receptor-Ca^{2+} release channel complex of skeletal muscle sarcoplasmic reticulum: Evidence for a cooperative coupled, negatively charged homotetramer. *J. Biol. Chem.* **264:**16776–16785

Marks, A.R., Fleischer, S., Tempst, P. 1990. Surface topography analysis of the ryanodine receptor/junctional channel complex based on proteolysis sensitivity mapping. *J. Biol. Chem.* **265:**13143–13149

McLachlen, A.D. 1976. The 14-fold periodicity in α-tropomyosin and the interaction with actin. *J. Mol. Biol.* **103:**271–298

Mellgren, R.L., Lane, R.D. 1988. Myocardial calpain 2 is inhibited by monoclonal antibodies specific for small, noncatalytic subunit. *Biochim. Biophys. Acta* **954:**154–160

Mellgren, R.L., Repetti, A., Muck, T.C., Easly, J. 1982. Rabbit skeletal muscle calcium-dependent protease requiring millimolar Ca^{2+}. *J. Biol. Chem.* **257:**7203–7209

Meyer, W.L., Fischer, S.H., Krebs, E.G. 1964. Activation of skeletal muscle phosphorylase *b* kinase by Ca^{2+}. *Biochemistry* **3:**1033–1039

Otsu, K., Willard, H.F., Khanna, V.K., Zorzato, F., Green, N.H., MacLennan, D.H. 1990. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sacroplasmic reticulum. *J. Biol. Chem.* **265:**13472–13483

Penner, R., Neher, E., Takeshima, H., Nishimura, S., Numa, S. 1989. Functional expression of the calcium release channel from skeletal muscle ryanodine receptor DNA. *FEBS Lett.* **259:**217–221

Rardon, D.P., Cefali, D.C., Mitchell, R.D., Seiler, S.M., Hathaway, D.R., Jones, L.R. 1990. Digestion of cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles with calpain II. *Circ. Res.* **67:**84–96

Rogers, S., Wells, R., Rechsteiner, M. 1986. Amino acid sequences common to rapidly degraded proteins: The pest hypothesis. *Science* **234:**364–368

Seiler, S., Wegener, A.D., Whang, D.D., Hathaway, D.R., Jones, L.R. 1983. High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum bind calmodulin, are phosphorylated and are degraded by Ca^{2+} activated protease. *J. Biol. Chem.* **257:**8550–8557

Solaro, R.S., Pang, D.C., Briggs, F.N. 1971. The purification of cardiac myofibrils with Triton X-100. *Biochim. Biophys. Acta* **245:**259–262

Takeshima, H., Nishimura, S., Matsumoto, R., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., Numa, S. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature* **339:**439–445

Thieleczek, R., Mayr, G.W., Brandt, N.R. 1989. Inositol polyphosphate-mediated repartitioning of aldolase in skeletal muscle triads and myofibrils. *J. Biol. Chem.* **264:**7349–7356

Walker, J.E., Saraste, M., Runwick, M.J., Gay, N.J., 1982. Distinctly related sequences in the α and β subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBOJ.* **1:**945–951

Wang, K.K.W., Villalobo, A., Roufogalis, B.D. 1989. Calmodulin-binding proteins as calpain substrates. *Biochem. J.* **262:**693–706

Yoshimura, N., Murachi, T., Heath, R., Kag, J., Jasani, B., Newman, G.R. 1986. Immunogold electro-microscopic localisation of calpain I in skeleton muscle of rats. *Cell Tissue Res.* **244:**265–270

Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G., MacLennan, D.H. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sacroplasmic reticulum. *J. Biol. Chem.* **265:**2244–2256

Received 23 August 1991; revised 11 December 1991