Protein p67
A Calcium-Binding Protein Localized at the Sarcolemma of Secretory Atrial Myocytes

Hiroshi Iida, Hideo Nishitani, and Yasuburo Shibata

Bovine heart 67-kd protein (p67) was coisolated with calpain I complex by cycles of Ca\(^{2+}\)-dependent precipitation followed by solubilization with EGTA-containing buffer. Using affinity-purified anti-p67 antibody and anti-p36 (36-kd subunit of calpain I) antibody, we examined the localization of the two proteins in secretory atrial myocytes and other endocrine tissues of adult rats. Immunofluorescence microscopy showed that p67 was expressed both in the atrial myocytes in situ and in cultured atrial myocytes in which we failed to detect p36 and that p67 appeared to be closely associated with the cell surface. We also found that p67 was colocalized with p36 in the thyroid follicle epithelium and zona reticularis of the adrenal gland. On the other hand, neither p67 nor p36 was detectable in pancreas islet cells. Immunoelectron microscopy revealed that p67 was localized at the sarcolemma in the atrial myocytes in situ. The p67, which was shown to be a globular molecule with a diameter of 18–25 nm by a low-angle rotary shadowing method, bound radioactive Ca\(^{2+}\) on a nitrocellulose membrane. The results suggest that Ca\(^{2+}\)-binding proteins expressed in endocrine cells seem to vary from tissue to tissue and that p67 may function in Ca\(^{2+}\)-mediated events at the plasma membrane of secretory atrial myocytes and some types of endocrine cells expressing this protein. (*Circulation Research* 1992;70:370–381)

The exocytotic fusion of membrane vesicles with the plasma membrane probably occurs in all cells and is involved in the release of secretory products from vesicles. In the case of endocrine cells specialized to secrete hormones in a regulated pathway, exocytosis is triggered on cell activation by an intracellular signal that is often a transient rise in the concentration of intracellular calcium. Atrial natriuretic peptide (ANP), a hormone that is a potent diuretic, natriuretic, and vasorelaxant polypeptide,\(^1\) is secreted from atrial myocytes. Exocytosis of ANP is triggered by either an influx of extracellular calcium, release of calcium from intracellular storage, or both,\(^2\)–\(^5\) although the mechanism by which calcium regulates ANP release from the atrial myocytes remains to be defined.

The importance of calcium in the regulation of exocytosis has led to a search for putative calcium receptor proteins involved in mediating the interaction and fusion of secretory vesicle with the plasma membrane. Recently, a new family of calcium receptor proteins or Ca\(^{2+}\)-regulated phospholipid-binding proteins has been identified. Of these proteins, which have been given the generic name annexin,\(^6\) calpain I seems to be a leading candidate for a crucial Ca\(^{2+}\)-receptor that might promote contact between secretory vesicles and the plasma membrane.\(^7\)

In the present study, calpain I and a coisolated 67-kd protein (p67) were obtained from bovine lung or heart by cycles of Ca\(^{2+}\)-dependent precipitation followed by EGTA resolubilization. Using affinity-purified antibodies to p67 and the 36-kd subunit of calpain I (p36), we examined the distribution of both antigens in atrial myocytes and other endocrine tissues of adult rats by immunocytochemical techniques. The results showed that both atrial myocytes in situ and cultured atrial myocytes expressed a significant amount of p67 but did not contain a detectable amount of p36. The p67 antigen in the atrial myocytes was found to be closely associated with the sarcolemma of the cells. Although neither p36 nor p67 was a ubiquitous protein in rat endocrine cells, p67 was colocalized with p36 in some types of cells of endocrine tissues that were examined. Amino acid composition, Ca\(^{2+}\)-binding property, and molecular shape of the bovine heart p67 were also shown in the present study.

**Materials and Methods**

**Purification of Proteins**

Calpain I complex was isolated by a modification of the procedure previously described.\(^8\) Bovine lungs

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or hearts were homogenized for 2 minutes in a Waring blender in 50 mM Tris-Cl (pH 7.3), 2 mM MgCl₂, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF); then, 10 mM solid CaCl₂ was added and the suspension was homogenized for another 2 minutes. After the suspension was stirred for 20 minutes at 4°C, it was centrifuged at 3,000g for 15 minutes and the supernatant was further centrifuged at 25,000g for 1 hour. The pelleted material from the second centrifugation was suspended in buffer A (10 mM imidazole, 1 mM CaCl₂, 0.5 mM DTT, and 0.1 M KCl [pH 7.3]) containing 1% Triton X-100 and stirred for 20 minutes at 4°C. After centrifugation at 25,000g for 1 hour, the pellets were washed twice more times in buffer A by resuspension and centrifugation. The pelleted proteins were extracted with 10 mM EGTA in buffer A by stirring for 30 minutes at 4°C. The solution was centrifuged at 100,000g for 1 hour, and the soluble proteins were dialyzed against 10 mM imidazole, 1 mM CaCl₂, and 0.5 mM DTT (pH 7.3).

Precipitated proteins were collected by centrifugation at 30,000g for 30 minutes and redissolved in 5 ml buffer B (20 mM sodium phosphate, 0.5 M NaCl, 3 mM EGTA, 0.5 mM DTT, and 1 mM Na₃[PO₄] [pH 7.0]) and applied to a 2.5×100 cm column of Sephadex G-150 equilibrated and run in the same buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fractions showed that calpactin I complex was coisolated with p67 (see Figure 1A). Fractions containing calpactin I complex and p67 were pooled, dialyzed against 10 mM imidazole (pH 7.3), 1 mM EGTA, 0.5 mM DTT, and 1 mM Na₃[PO₄], and applied to a DE-52 column (Whatman Inc., Clifton, N.J.) equilibrated in the buffer. Calpactin I complex came through this column, whereas p67 was retained and eluted with 0–500 mM NaCl gradient. Purified p67 was pooled, dialyzed against 0.5 mM DTT, and stored at −80°C. The calpactin pools were adjusted to 20 mM sodium acetate (pH 5.6), and the solution was applied to a CM-52 column (Whatman) and eluted with 1 M NaCl. The purified calpactin I complex was dialyzed against 0.5 mM DTT and stored at −80°C.

**Limited Proteolysis of Calpactin I Complex**

A 1.5-mg sample of calpactin I as a complex with a light chain in 2 ml of 10 mM Tris-Cl and 1 mM DTT (pH 7.3) was treated with chymotrypsin (10 μg/ml) for 60 minutes. Digestion was terminated by addition of 1 mM PMSF, and the sample was applied to 4 ml DE-52 cellulose column equilibrated in the same buffer. The 33-kd core protein of calpactin I complex that passed through unimpeded was dialyzed against 0.5 mM DTT and stored at −80°C.

Protein concentration was determined according to the method of Bradford using y-globulin as a standard.

**EGTA Extraction of Rat Hearts**

Rat hearts were homogenized at 4°C in a buffer containing 40 mM Tris-Cl, 10 mM EGTA, 1% Triton X-100, 0.5 mM DTT, and 1 mM PMSF (pH 7.0). After centrifugation at 3,000g for 20 minutes, the supernatant was further centrifuged at 100,000g for 1 hour. After the second centrifugation, the supernatant was subjected to SDS-PAGE.

**Preparation of Antibodies**

Japanese White rabbits were immunized on three occasions at 2-week intervals with 0.4 mg purified calpactin I complex or p67 mixed with Freund’s adjuvant (complete for the first injection and subsequently incomplete). The proteins were injected subcutaneously at multiple sites on the back. Antisera were collected 10 days after the final injection. Affinity purification of antibodies was achieved with nitrocellulose blots by the method of Smith and Fisher. Affinity-purified anti-p36 antibody and anti-p67 antibody were concentrated and stored at −40°C in the presence of 1% albumin. The affinity-purified antibodies were used for immunoblot analysis and immunocytochemistry in the present study.

**Electrophoresis and Immunoblotting**

Proteins were separated by electrophoresis on 12% or 15% SDS polyacrylamide gels following the procedure of Laemmli. Immune blotting was performed by one-dimensional electrophoresis followed by electrophoretic transfer to nitrocellulose sheets. Nitrocellulose sheets were stained with either affinity-purified anti-p36 antibody or anti-p67 antibody followed by horseradish peroxidase–labeled goat anti-rabbit immunoglobulin G (IgG; Bio-Rad Laboratories, Richmond, Calif.), and the localization of peroxidase was detected by the reaction using diaminobenzidine.

**Cell Culture**

Primary cultures of atrial myocytes on 13-mm glass coverslips coated with laminin (10–20 μg per dish; Sigma Chemical Co., St. Louis, Mo.) were prepared from the hearts of ether-anesthetized 250–300-g Wistar rats as previously described. The cells were cultured at 37°C with medium 199 supplemented with 10% fetal bovine serum in an incubator. The cells were used for experiments on days 7 and 8 of culture.

**Immunofluorescence Microscopy**

Endocrine organs of adult rats were frozen in liquid N₂–cooled OCT compound (Tissue Tek, Miles Inc., Elkhart, Ind.). Sections (5–10 μm) were mounted on slide glass, air-dried, fixed for 10 minutes in acetone at −20°C, and then stained for 1 hour with either affinity-purified anti-p36 antibody or anti-p67 antibody. After several washes in phosphate-buffered saline (PBS), the specimens were incubated with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate or rhodamine B isothiocyanate (FITC) (Tago, Inc., Burlingame, Calif.), washed in PBS, and examined with an Olympus BH-12 fluorescence microscope, as described previously. Preimmune rabbit serum instead of the primary antibody was used as a control. Cultured atrial myocytes were fixed for 10
minutes in methanol/acetone (1:1) at −20°C, air-dried, and stained with antibodies as described above.

For adsorption studies, 10 μg anti-p36 antibody was incubated at 4°C overnight with either 0 or 15 μg calpactin I, and 10 μg anti-p67 antibody was similarly treated with either 0 or 10 μg p67 antigen. The supernatants obtained by centrifugation were used for immunofluorescence studies in frozen sections of small intestine. Staining patterns of the antibodies incubated with no antigens were similar to those shown in Figures 2A and 2B.

Subcellular Localization of p67 Antigen on Ultrathin Sections

Auricles of adult Wistar rats were fixed for 3 hours in periodate lysine paraformaldehyde fixative containing 1% paraformaldehyde at 4°C. The samples were washed in PBS, incubated for 20 minutes with 50 mM NH₄Cl in PBS, dehydrated in a graded series of dimethylformamide, and then embedded in Lowicryl K4M (Polysciences, Inc., Warrington, Pa.) at 4°C. After the samples were blocked with 5% normal goat serum, thin sections were incubated for 90 minutes with affinity-purified anti-p67 antibody followed by incubation for 60 minutes with 10-nm colloidal gold–labeled goat anti-rabbit IgG (Janssen Life Science Products, Beerse, Belgium). Preimmune rabbit serum instead of the primary antibody was used as a control. After the specimens were washed with PBS, they were fixed for 3 minutes with 2% glutaraldehyde in PBS, washed extensively with water, and stained with uranyl acetate and lead citrate. The specimens were examined in a JEOL 2000-EX electron microscope.

Amino Acid Analysis

Purified bovine heart p67 was extensively dialyzed against distilled water and hydrolyzed at 110°C for 24 hours. Amino acid composition of the protein was analyzed with a Beckman DABS amino acid analysis system.

Radioactive ⁴⁵Ca²⁺-Binding Study

Radioactive ⁴⁵Ca²⁺ binding to proteins that were adsorbed to nitrocellulose membrane was performed by a modification of the procedure described by Maruyama et al. ¹³ Purified bovine heart p67 and γ-globulin (negative control) were adsorbed to nitrocellulose membranes at a protein concentration of 0.5–4 μg per spot. After the membranes were air-dried, they were washed with a buffer containing 60 mM KCl, 0.5 mM MgCl₂, and 10 mM imidazole-HCl (pH 7.1), incubated for 1 hour with the buffer containing ⁴⁵Ca²⁺ (3 mCi/ml; New England Nuclear Corp., Boston, Mass.), and washed with water for 5 minutes. Autoradiographs of ⁴⁵Ca²⁺-labeled proteins on the membranes were obtained by exposure of the dried membranes to Kodak XAR-5 x-ray films for 18 hours. Some protein-adsorbed membranes were stained with amido black.

Low-Angle Rotary Shadowing Method

Purified bovine heart p67 was suspended in 50% glycerol, 10 mM Tris-Cl (pH 7.3), and 1 mM EGTA at a protein concentration of 80 μg/ml and then sprayed on freshly cleaved mica flakes. The samples were dried under vacuum at room temperature and rotary shadowed with platinum at an angle of 2–5° followed by carbon. The specimens were examined in a JEOL 2000-EX electron microscope.

Results

Purification of Calpactin I Complex and 67-kd Protein

Cycles of Ca²⁺-dependent precipitation followed by EGTA resolubilization from an extract of bovine lung resulted in isolation of a limited number of proteins with molecular weights of 67,000, 36,000–32,000, and 10,000 on SDS-PAGE. Gel filtration of this material in high salt, low Ca²⁺-containing buffer allowed the separation of these proteins (Figure 1A). Calpactin I complex (p36,p102, fraction 15–18 in Figure 1A) that passed through the Sephadex G-150 column was partially overlapped with a minor protein with a molecular weight of 67,000. Separation of calpactin I complex from the 67-kd protein was achieved by ion-exchange chromatography as described in “Materials and Methods.” An essentially pure form of calpactin I complex was obtained by eluting the protein retained in the CM-52 column with 1 M NaCl (Figure 1B, lane 2). The observation that treatment of the purified protein with chymotrypsin yielded a 33-kd protein (Figure 1B, lane 3), which should correspond to the core protein of the calpactin I heavy chain, ¹⁴ confirmed for us that the purified protein was calpactin I complex. Starting with 720 g tissue, we could isolate 13.2 mg of calpactin I complex, whereas only 0.4 mg of the 67-kd protein was obtained.

Several proteins with similar molecular masses were obtained from bovine heart extracts by the cycles of Ca²⁺-dependent precipitation followed by EGTA resolubilization. SDS-PAGE analysis of this material showed that it contained approximately the same amount of calpactin I heavy chain (p36) and a protein of Mr 67,000 (p67) (Figure 1C, lane 2, and Figure 1D, lane 2). Starting with 2,300 g bovine heart tissue, we could obtain 8.5 mg p67. The purified bovine heart p67 (Figure 1C, lane 4) and calpactin I complex from bovine lung were used to immunize rabbits to obtain antibodies to these proteins.

Characterization of the Antibodies to p36 and p67

Characterization of the antibodies that we obtained was defined by both immunoblot analysis and immunofluorescence microscopy. After blot-affinity purification, anti-p36 antibody reacted selectively with the 36-kd subunit of calpactin I but not at all with p67 (Figure 1D, lane 3). Antibody to p67 showed strong immunoreactivity with the bovine heart p67 but not with calpactin I (Figure 1D, lane 4).
Likewise, on the nitrocellulose paper to which EGTA-extracted whole proteins of rat hearts were transferred, anti-p36 antibody and anti-p67 antibody labeled a polypeptide band in the position of the 36,000 subunit of calpactin I and p67, respectively (Figure 1D, lanes 6 and 7).

We then carried out immunofluorescence microscopy to examine the specificity of the affinity-purified antibodies. Previous studies have shown that the brush border of porcine\textsuperscript{15} and rodent\textsuperscript{16} small intestine contained calpactin I or p36. We therefore chose rat small intestine for immunofluorescence microscopy. Frozen sections of rat small intestine stained with either anti-p36 antibody or anti-p67 antibody are shown in Figure 2. The antigen detected by anti-p36 antibody was localized at the brush border (Figure 2A) and serous membrane (not shown), whereas anti-p67 antibody displayed strong affinity for lamina propria cells (Figure 2B) and smooth muscle cells (not shown). The intensity of immunofluorescence staining was greatly diminished by prior incubation of the antibodies with purified antigens (Figures 2C and 2D). Taken together, these results indicate that the antibodies to p36 and p67 were specific for the two distinct proteins, showing no detectable cross-reaction in both immunoblot analysis and immunofluorescence microscopy.

**Localization of p36 and p67 in Atrial Myocytes in Situ and Cultured Atrial Myocytes**

Immunofluorescence microscopy was carried out to examine the distribution of p36 and p67 in both rat atrial tissues and cultured atrial myocytes. The primary culture established from adult rat auricles
comprises atrial myocytes (about 90% of cells) and minor contaminated cells such as fibroblasts and endothelial cells. On frozen sections of atrial tissues, the atrial myocytes were not stained with anti-p36 antibody, whereas epicardium, endocardium, and nonmuscle cells present in the connective tissue between the myocytes were intensely stained with the antibody (Figure 3A). In contrast, p67 was concentrated at the cell periphery of the atrial myocytes (Figure 3B). Likewise, cultured atrial myocytes, which were identified as a major constituent in the culture by staining with anti-ANP antibody (Figure 3C), did not contain detectable amounts of p36, whereas both contaminated fibroblasts and endothelial cells were intensely stained with the antibody (Figures 3D and 3E). In a group of endothelial cells, increased staining was often seen at the cell-to-cell contact face (Figure 3E). In contrast, fluorescence labeling for the p67 antigen caused bright staining of cultured atrial myocytes that possessed cell processes (Figure 3F), and the staining was often accentuated at the cell surface and the region where neighboring cells abut (Figure 3G). Contaminated fibroblasts and endothelial cells contained little, if any, p67.

**Subcellular Localization of p67 in the Atrial Myocytes**

Subcellular localization of the p67 antigen in the atrial myocytes in situ was examined by immunogold electron microscopy. Most of gold labels were observed at the cell-to-cell contact area (Figure 4A) and the cell surface (Figure 4B). At high magnification, gold labels appeared to be closely associated with the sarcolemma (Figure 4C). The p67 was also found at the intercalated disk region (Figure 4D), whereas gap junction–like plaques where two plasma membranes were apparently closely apposed were devoid of gold labels (not shown). Few gold particles were observed on other cell organelles, such as nuclei, mitochondria, secretory granules, and myofibrils (Figures 4A–4D). In sections incubated with
FIGURE 3. Immunofluorescence localization of the 36-kd subunit of calpactin I (p36) and a 67-kd protein (p67) in frozen sections of rat atrial tissues (panels A and B) and cultured cells established from rat auricles (panels C–G). In frozen sections of atrial tissues, immunostaining with anti-p36 antibody (panel A) is seen in cells of connective tissue and endocardium, whereas localization of p67 is restricted to the cell periphery of atrial myocytes (panel B). Cultured atrial myocytes, which are recognized as a major cell type in culture by staining with anti-atrial natriuretic peptide antibody (panel C), are not stained with anti-p36 antibody (panel D, arrows) but intensely stained with anti-p67 antibody (panels F and G). A fibroblast-like cell (panel D) and presumptive endothelial cells (panel E), which are contaminated cell types in culture, are positive for the p36 antibody. Bars: panels A–D and F, 50 μm; panels E and G, 10 μm.
Localization of a 67-kd protein (p67) on ultrathin sections of atrial tissue at the electron microscopic level. Immunogold electron microscopy shows that 10-nm gold particles are mainly localized at the cell-to-cell contact area (panel A), cell surface (panel B), and intercalated disk region (panel D). At high magnification (panel C), gold particles are closely associated with the sarcolemma. Nucleus (N), mitochondria (M), myofibrils (MF), and secretory granules (S) are devoid of specific labeling. Bars: panel A, 0.5 μm; panels B and D, 0.25 μm; panel C, 0.2 μm.

Distribution of p36 and p67 in Rat Endocrine Tissues

To examine whether p67 is a ubiquitous protein in endocrine cells, as well as to compare the distribution of p67 with that of p36 in endocrine tissues, we studied the distribution of the two proteins in pancreas, thyroid gland, and adrenal gland of adult rats by immunofluorescence microscopy on frozen sections.

In pancreas, both anti-p36 antibody and anti-p67 antibody failed to stain islet cells and exocrine acinar cells, whereas fibroblast-like cells in connective tissue contained a detectable amount of both p36 and p67 (Figures 5A and 5B). In addition, endothelial cells (Figure 5A) and epithelial cells of acinar ducts (not shown) expressed p36 and p67, respectively. In thyroid gland, anti-p36 antibody displayed strong affinity for the cell periphery of the follicle epithelium; the luminal surface of the follicles was most intensely stained (Figure 5C). Fibroblasts in the connective tissue were also positive for the antibody. The luminal surface of the follicles was also stained with anti-p67 antibody, but p67-associated fluorescence at
the luminal surface was not as intense as that of p36 (Figure 5D). In adrenal cortex, the distribution of p36 and p67 was strikingly similar; both antigens were found to be present in capsule, blood vessels, and endocrine cells in zona reticularis (Figures 5E and 5F). In the adrenal medulla, p36 was mainly found in endothelial cells of blood vessels (Figure 5E), whereas p67 was detected in connective tissue surrounding a part of medulla cells or cell periphery of them but not at all in endothelial cells of blood vessels (Figure 5G).

Figures 5H–5K show control micrographs of endocrine tissues stained with preimmune serum followed by FITC-conjugated secondary antibodies. No staining was observed except that small dotlike fluorescence was seen in pancreas tissues (Figure 5I), which might be due to autofluorescence because the dotlike fluorescence was seen in the tissue sections without immunostaining.

Characterization of the Bovine Heart p67

Amino acid composition of p67. A 67-kd Ca

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+-binding protein, calcimedin, has been shown to be expressed in both cardiac myocytes and smooth muscle cells like the p67 reported here. We therefore intended to compare the amino acid composition of p67 (Table 1) with that of calcimedin reported by Moore. Identity is not supported by the analysis of amino acid composition, which reveals that the amounts of histidine, isoleucine, and leucine of p67 were appreciably different from those of 67-kd calcimedin.

Ca

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+-binding experiment. The bovine heart p67 could be isolated by Ca

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+-dependent precipitation followed by EGTA resolubilization as reported for many Ca

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+-binding proteins (see “Discussion”), suggesting that p67 might be a Ca

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+-binding protein. To obtain more direct proof that p67 is indeed a Ca

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+-binding protein, p67 and γ-globulin (control) that were adsorbed to nitrocellulose membranes were probed with 45Ca

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followed by autoradiography. To reduce nonspecific binding of 45Ca

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to the membranes, incubation of the membranes with 45Ca

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was carried out in the presence of magnesium ions. The result showed that p67 did bind 45Ca

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, whereas γ-globulin (control) remained negative (Figure 6). It was estimated that at least 2 μg per spot of p67 could be detected on the membranes.

Low-angle rotary shadowing study. Molecular shape of the purified bovine heart p67 was examined by a low-angle rotary shadowing method. On freshly cleaved mica, rotary shadowed bovine heart p67 appeared as a globular molecule with a diameter of 18–25 nm, and we saw an indentation within at least some molecules (Figure 7). Other molecules, however, did not show such appreciable indentation, probably because the molecules were randomly attached to the surface of mica, which concealed the indentation. The same result was obtained when salts such as Tris and EGTA were removed from the sample solution.

Discussion

The new family of Ca

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+-dependent membrane-binding proteins, which has been given the generic name annexin, has been identified in various cells and tissues. These proteins can be classified according to their molecular masses into three major groups of 32, 36, and 68 kd, which share conserved domains. However, the species and tissues from which they were derived are so varied that their exact relations are still not clear. The proteins of the annexin family, because of their localization at cell periphery and their chemical nature, have been expected to be involved in the plasma membrane–associated processes or the interaction between the plasma membrane and cytoskeletons. Of these proteins, calpactin I might be a leading candidate for a crucial receptor for intracellular Ca

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++, which promotes membrane fusion in exocytosis. In addition, direct involvement of the calpactin I complex in cross-linking between secretory granules and the plasma membrane has been suggested by quick-freeze, deep-etch electron microscopy.

Calpactin I, a major cellular tyrosine kinase substrate, is also a substrate for protein kinase C both in vivo and in vitro. Activation of protein kinase C has been reported to stimulate ANP release from isolated perfused hearts and cultured atrial myocytes. Therefore, we expected that calpactin I might be expressed in the atrial myocytes and involved in exocytosis in the cells. However, we failed to detect the protein in the atrial myocytes, which might be due to the lack or low level of the protein expressed in the cells. Instead, p67, which was coisolated with calpactin I from bovine hearts, was found to be expressed in the atrial myocytes.

The p67 purified from bovine hearts shares several properties with calpactin I. First, p67, like calpactin I, can be isolated from tissue extract by Ca

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+-dependent precipitation followed by EGTA resolubilization, as shown in Figure 1. Second, p67 is closely associated with the plasma membrane (sarcolemma) of the atrial myocytes, as reported for calpactin I in some types of cells. Third, p67 was found to be colocalized with calpactin I in thyroid follicle epithelium and zona reticularis of adrenal gland of adult rats. Fourth, p67 is probably a Ca

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+-binding protein like calpactin I. Furthermore, we observed that antigen immunoreactive with anti-spectrin antibody was localized at the sarcolemma of the atrial myocytes (data not shown), as reported for ventricular myocytes. The result suggests that p67 is at least partially colocalized with spectrinlike protein, as reported for calpactin I in fibroblasts and intestinal epithelial cells. Further studies are required to define the function of p67 in the atrial myocytes, but these results encourage us to speculate that p67 seems to be closely related to calpactin I and that p67 might be one of the candidates that are involved in Ca

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+-regulated membrane-associated processes or the linkage between the sarcolemma and cytoskeleton-
tons in the atrial myocytes. Because both Ca\(^{2+}\) influx from calcium channels at the sarcolemma and Ca\(^{2+}\) release from sarcoplasmic reticulum stimulate ANP release from the atrial myocytes\(^{2-5}\) and because p67 is probably a Ca\(^{2+}\)-binding protein located at the sarcolemma, it might be assumed that p67 mediates Ca\(^{2+}\)-induced interaction between atrial secretory granules and the sarcolemma, by which p67 could modulate ANP release.

Several proteins with molecular masses of 67–73 kd, which seem to be members of the annexin proteins, have been isolated from various tissues and cells, although their physiological functions have not yet been defined.\(^{21}\) These include human lymphocyte p68,\(^{32,33}\) 67-kd calelectrin,\(^{34}\) bovine intestinal 73-kd protein,\(^{8}\) porcine liver 68-kd protein III,\(^{35}\) and human placental 68 kd I and II proteins,\(^{36}\) all of which can be coisolated with a limited number of proteins, such as calpactin I, by Ca\(^{2+}\)-dependent precipitation followed by EGTA resolubilization, as shown in the case of bovine heart p67 in the present study. Recent amino acid sequence data indicate that of these annexin-like proteins, lymphocyte p68 is the same as 67-kd calelectrin.\(^{37}\) On the other hand, immunocytochemical studies have shown that another Ca\(^{2+}\)-binding protein, 67-kd calcimedin, seems to be distinct from 67-kd calelectrin and lymphocyte p68.\(^{18,19}\)

Identification of other proteins, however, has not yet been clearly defined. Identity of bovine heart p67 to any of the annexin family proteins reported so far must wait until amino acid sequence of p67 becomes available.

**Figure 5.** Localization of the 36-kd subunit of calpactin I (p36) and a 67-kd protein (p67) in various rat endocrine tissues by immunofluorescence staining. Frozen sections of pancreas (panels A and B), thyroid gland (panels C and D), and adrenal gland (panels E–G) are incubated with either affinity-purified anti-p36 antibody (panels A, C, and E) or anti-p67 antibody (panels B, D, F, and G) followed by fluorescein isothiocyanate– or rhodamine B isothiocyanate (RITC)–labeled secondary antibodies. In pancreas, neither islet cells (PI) nor exocrine acinar cells contain detectable amounts of both antigens (panels A and B). Thyroid follicle epithelium is stained with anti-p36 antibody (panel C). The luminal surfaces of the follicles are also stained with anti-p67 antibody (panel D). In adrenal cortex, both anti-p36 and anti-p67 antibodies display strong affinity for cells in zona reticularis (panels E and F). Endothelial cells of blood vessels of adrenal medulla are stained with anti-p36 antibody (panel E), whereas the p67 antigen is localized in connective tissue surrounding a part of the medulla cells or cell periphery (panel G, arrows). Panels H–K show control micrographs of auricles (panel H), pancreas (panel I), thyroid glands (panel J), and adrenal glands (panel K), which are stained with preimmune serum followed by RITC-conjugated secondary antibodies. Bars: panels A–D and G–J, 50 \(\mu\)m; panels E, F, and K, 100 \(\mu\)m.
TABLE 1. Amino Acid Composition of p67

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<th>mol %</th>
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The present study showed that in the rat endocrine organs examined, calpain I was expressed in thyroid follicle epithelium and zona reticularis of adrenal gland but was not detectable in the atrial myocytes and pancreas islet cells. Greenberg et al.13 also failed to detect 34-kd tyrosine kinase substrate, which should be identical to p36, in chicken pancreas islet cells. Therefore, it is unlikely that calpain I is a ubiquitous protein in endocrine cells although we cannot rule out the possibility that the failure to detect calpain I in atrial myocytes and pancreas islet cells is due to the very low level of the protein expressed in these cells. The significance of colocalization of calpain I and p67 in thyroid follicle epithelium and zona reticularis of adrenal gland is not clear at present, but there is a possibility that these two distinct proteins cooperate in endocrine cells.

It has been reported that p36 expression in some types of cells is turned on when cells are put into culture.16,31 This makes it important to examine whether cells for experiments express p36 both in situ and in culture. The fact that both atrial myocytes in situ and cultured atrial myocytes express p67 but do not contain a detectable amount of p36 makes it clear that the expression of p67 in the cells is not altered by "culture" and the expression of p36 is not turned on by cultivating the cells in vitro. Cultured atrial myocytes that have been used for pharmacological and morphological experiments, therefore, should be a useful system to study the physiological function of p67.

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