A Plasma Membrane Integral Sialoglycoprotein (Sgp 130) Molecularly Distinguishes Nonjunctional Dense Plaque Sites of Microfilament Attachment

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Abstract. An integral sialoglycoprotein with M_r ~130,000 (Sgp 130) and highest expression in adult chicken gizzard smooth muscle has been recently identified as an excellent candidate for classification as a plasma membrane protein natively associated (directly or indirectly) with actin microfilaments (Rogalski, A. A., and S. J. Singer, 1985, *J. Cell Biol.*, 101:785–801). In this study, the relative in situ distributions of the Sgp 130 integral species (a designation that also includes non-smooth muscle molecular forms) and the peripheral protein, vinculin, have been simultaneously revealed for the first time in selected cultured cells and tissues abundant in microfilament–membrane attachment sites, particularly, smooth and cardiac muscle. Specific antibody probes against Sgp 130 (mouse mAb 30B6) and vinculin (affinity-purified rabbit antibody) were used in double indirect immunofluorescent and immunoelectron microscopic experiments. In contrast to the widespread distributions of vinculin at microfilament–membrane attachment sites, Sgp 130 has been shown to exhibit striking site-specific variation in its abundancy levels in the plasma membrane. Sgp 130 and vinculin were found coincidentally concentrated at focal contact sites in cultured chick embryo fibroblasts and endothelial cells, membrane dense plaques of smooth muscle, and sarcolemma dense plaque sites overlying the Z line in cardiac muscle. However, at the fascia adherens junctional sites of cardiac muscle where vinculin is sharply confined, Sgp 130 was immunologically undetectable in both intact and EGTA-uncoupled tissue. This latter result was confirmed with immunoblotting experiments using isolated forms of the fascia adherens.

The double immunolabeling studies of this report establish Sgp 130 as a major integral protein component of nonjunctional membrane dense plaque structures and raise the possibility that the 130-kD integral sialoglycoprotein (Sgp 130) and vinculin assume stable transmembrane associations at these particular microfilament–membrane attachment sites. Nonjunctional dense plaques are further suggested to be a molecularly distinct class of plasma membrane structures rather than a subgroup of adherens junctions. Our data also support a hypothesis that Sgp 130 is involved in plasma membrane force coupling events but not in junctional-related cell–cell coupling.

The role of the cytoskeleton in spatially and functionally organizing the cytoplasm of nonerythrocyte cell types has been a subject of broad experimental interest. An important and challenging issue in this area concerns the mechanisms by which cytoskeletal elements, particularly actin microfilaments, assume both specific associations with the plasma membrane and key roles in basic cellular processes (20, 22, 29, 31, 42, 45, 52).

Numerous studies have shown actin microfilament–plasma membrane associations to be either (*a*) highly specialized at discrete attachment sites, such as, for example, the membrane dense plaque of smooth muscle (16, 18), the fascia adherens of cardiac muscle (48) or stereocilia of cochlea hair cells (45), or (*b*) less well-defined structurally and subject to modulation in a series of cellular events that are transient (i.e., cytokinesis) (43) or developmentally regulated (i.e., myofibril attachment to the sarcolemma) (13).

Microfilament–plasma membrane associations also appear to be functionally specialized at sites of cell–cell adhesion and at sites of cell contractility where cytoskeletal activities are translated into dynamic cellular events of cytokinesis, endocytosis, and cell movement.

We are interested in studying the relative in vivo arrangements of specific membrane proteins as one approach for revealing some of the molecular details that underly functional specializations of microfilament–membrane attachment sites. That specific subsets of interrelated, peripheral, and integral membrane proteins contribute to and/or regulate the function of microfilament–membrane attachment sites is a testable hypothesis that we have begun to explore here by investigating the in situ distributions of specific peripheral and integral membrane proteins, namely vinculin (17) and a recently discovered 130-kD integral sialoglycoprotein (Sgp 130)[1] (40).

1. *Abbreviations used in this paper*: CSAT, cell substratum attachment; Sgp 130, 130-kD integral sialoglycoprotein.

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As chemically distinct, actin-related proteins, vinculin and Sgp 130 are among the candidates of membrane-associated species (3, 23, 25, 36, 38) that may coordinately participate in the linkages of microfilaments to the plasma membrane.

Vinculin had been originally proposed by Geiger and coinvestigators (17) to function as a peripheral protein involved in the attachment of the termini of microfilament bundles to membranes. Despite evidence for the distribution of vinculin relative to actin microfilaments (17-20), as well as other cytoskeletal-related proteins, such as alpha-actinin (18) and talin (3), at junctional (18, 48) and nonjunctional (9, 37) membrane sites, the molecular/functional basis for vinculin interactions with actin microfilaments and the plasma membrane are still largely unresolved. The results of several recent studies have indicated, however, that vinculin may have specific binding sites for alpha-actinin (10) and talin (4, 27), and that the association of vinculin with the plasma membrane may be actin independent in nature (1, 15, 53).

The 130-kD sialoglycoprotein, as specifically recognized by monoclonal antibody 30B6, is an integral membrane species closely correlated with microfilament distributions in diverse cultured cell types at both intercellular and cell–substrate contact sites (40). While the expression of this 130-kD membrane species is at its highest level in adult chicken gizzard smooth muscle, all of its molecular forms will now be collectively referred to as Sgp 130. Recent biochemical studies (Rogalski, A. A., manuscript in preparation) indicate that Sgp 130 as a sialylated molecule is charge heterogeneous and assumes at least three tissue-specific isoforms that are developmentally regulated. The cell substratum attachment (CSAT) fibronectin receptor glycoprotein complex (26, 30) may have secondary associations with one of the Sgp 130 isoforms.

In this paper we focus on the possible interrelationships between the Sgp 130 integral species and the peripheral protein, vinculin, by studying their relative in situ topologies in cultured cells and in tissues abundant with microfilament–membrane attachment sites. For some of these experiments we applied the technology of double indirect immunoelectron microscopy (14, 44, 46, 47), using an anisometric iron-dextran secondary antibody conjugate (Imposil) to indirectly label affinity-purified rabbit antibodies to vinculin, and the isometric ferritin secondary antibody conjugate to indirectly label bound mAb 30B6 to Sgp 130. A significant new finding of these studies is the fact that there exist microfilament–membrane attachment site-specific differences in the abundancy levels of Sgp 130. Sgp 130 and vinculin are both demonstrated to be highly concentrated at the membrane dense plaque of gizzard smooth muscle and Z line attachment plaques to the sarcolemma of cardiac muscle. Yet, Sgp 130 is immunologically undetectable both in the in situ and isolated forms of the fascia adherens junctions of cardiac muscle intercalated discs even though vinculin is sharply confined and strongly associated with these membrane sites of microfilament attachment. These data suggest that Sgp 130 may assume stable transmembrane associations with vinculin at certain functionally specialized microfilament–membrane attachment domains, particularly nonjunctional plasma membrane dense plaque sites. A preliminary report of this work has been presented in abstract form (41).

Materials and Methods

Cell Culture

Primary cultures of chicken embryo fibroblasts were prepared from 8–10-d-old embryos. Trypsinized cells from 24–28-h primary cultures were subcultured to ~75% confluence on 22-mm glass coverslips in DME/F_{12} medium with 10% FCS. Endothelial-enriched cultures were derived by combined trypsinization and elastase treatment and gentle scraping of split-open aortas from 1-mo-old chickens. Cells were then cultured in DME/F_{12} media with 15% FCS supplemented with chicken embryo extracts. Endothelial cells were identified by metabolically labeling cultures with acetylated low density lipoprotein modified with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Molecular Probes, Junction City, OR) by the methods of Voyta et al. (51).

Primary and Secondary Immunolabeling Reagent Preparations

Three lines of evidence (immunoblotting, immunoaffinity chromatography, and immunoprecipitation) on the high affinity and specificity of the 30B6 mAb to the 130-kD integral sialoglycoprotein, Sgp 130, particularly in its nonreduced molecular mass form of 107 kD, have been presented in a previous report (40). Rabbit antibodies to DEAE-purified pig heart vinculin were affinity-purified on a Ultrogel AcA-22 (LKB Instruments, Inc., Rockville, MD) linked DEAE-purified chicken gizzard vinculin column. Antibody specificity was demonstrated by immunoblotting experiments whereby probing nitrocellulose replicas of SDS-PAGE separated crude gizzard extracts and purified vinculin preparations resulted in detection of only the 130-kD vinculin species. With 10 mM CHAPS gizzard detergent extracts, both vinculin and metavinculin (12) were recognized by the anti-vinculin antibodies. Affinity-purified, biotinylated $(FAb')_2$ fragments of goat anti-mouse IgG were obtained from Cooper Biochemical Inc., Malvern, PA, fluorescein-conjugated streptavidin from Amersham Corp., Arlington Heights, IL, and ferritin-conjugated streptavidin from Bethesda Research Laboratories, Gaithersburg, MD. Iron-dextran (Imposil) conjugated goat anti-rabbit IgG was prepared according to the methods of Dutton et al. (14) and was kindly provided by Dr. Anne Dutton, University of California, San Diego.

Simultaneously Mapping the Plasma Membrane Distributions of Sgp 130 and Vinculin

Double Immunofluorescence Labeling. (*a*) Coverslip cultures of chicken embryo fibroblasts and endothelial cells were fixed for 30 min at room temperature with 3% paraformaldehyde in PBS, pH 7.4 and rinsed three times with PBS/0.1 M glycine. In some cases fixed cultures were permeabilized by brief treatment with 0.1% Triton X-100 before immunolabeling with mAb 30B6 to Sgp 130 and/or affinity-purified rabbit antibodies to vinculin. The coverslip cell cultures were incubated with mAb 30B6 at 90 μg/ml and rinsed three times with PBS-0.1 M glycine (10 min each session) before incubation with affinity-purified, biotinylated $F(Ab')_2$ fragments of goat anti-mouse IgG (Cooper Biochemical, Inc.) at 20 μg/ml in PBS/0.1 M glycine, pH 7.4 followed by fluorescein-streptavidin (Amersham Corp.) at a 1:50 dilution in PBS/0.1 M glycine. After three rinses cells were incubated with rabbit anti-vinculin antibodies at 20 μg/ml followed by affinity-purified, lissamine rhodamine-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Avondale, PA) at 20 μg/ml. This sequence of antibody incubations was empirically found to give optimal double label detection of Sgp 130 and vinculin at microfilament attachment sites to the plasma membrane. If rabbit antibodies to vinculin and its secondary probe were first applied in the immunolabeling sequence, then subsequent detection of Sgp 130 with mAb 30B6 and its respective secondary reagents was reduced, apparently by inaccessibility problems induced by steric effects of the bound vinculin-related primary and secondary antibodies.

(*b*) Semithin (0.5 μm) frozen sections of gizzard and cardiac muscle tissues were prepared according to the methods of Tokuyasu (46, 47) from adult (6–8-wk-old) chickens. Tissues (gizzard smooth muscle and cardiac ventricular papillary muscle) were excised from adult birds under anesthesia (10 mg Numbutal per 100 g body wt), cut into small (~1 mm) blocks, and fixed for 30 min in 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and then stored in 0.5% paraformaldehyde in phosphate buffer at 4°C. The blocks were infused with 2.3 M sucrose, rapidly frozen in liquid N_2, and sectioned to ~0.5-μm thickness with a Sorvall MT-2 microtome

equipped for cryosectioning (Sorvall Instruments, Norwalk, CT). After a brief rinse in PBS/0.1 M glycine the sections were then pretreated with 2% gelatin in PBS/0.1 M glycine for 10 min before a series of incubations with mAb 30B6 and rabbit anti-vinculin as described above to indirectly label Sgp 130 and vinculin.

Immunofluorescent microscopy was performed with a Zeiss Photomicroscope III equipped with a vertical illuminator RS III for epifluorescence and a 63 × 1.4 NA planapochromat objective for Nomarski differential interference contrast. Interference reflection microscopy was performed by modifying the Zeiss Photomicroscope III according to the methods of Bereiter-Hahn et al. (2).

Double Immunoelectron Microscopic Labeling. Tissues were fixed and stored as outlined above for the semithin frozen sectioning experiments. In some experiments excised, beating hearts were perfused for 20 min with Ringer's physiological saline solution with 2 mM EGTA (8, 50) before fixation to promote junctional uncoupling at the intercalated disc region. All fixed tissue blocks were infused with 2.3 M sucrose and ultrathin sectioned at −90°C. Sections (50 nm–0.1 μm thick) mounted on glow-discharged, carbon/formvar-coated, high transmission hexagonal grids (Polaron Instruments, Cambridge, MA) were processed for double indirect immunodetection of Sgp 130 and vinculin as follows. Sections were first pretreated with 2% gelatin in PBS/0.1 M glycine and then successively reacted for 20 min with mAb 30B6 to Sgp 130 (3–10 μg/ml) followed by biotinylated $F(ab')_2$ fragments of goat anti-mouse IgG (20 μg/ml) and ferritin-streptavidin (1:20 dilution), and then rabbit anti-vinculin (20 μg/ml) followed by iron-dextran (Imposil) conjugated goat anti-rabbit IgG (15 μg/ml). Between each of these labeling steps specimens were washed for 10-min intervals by transferring grids through 10 drops of PBS/0.1 M glycine, pH 7.4. Sections were then positive-stained using the absorption methods of Tokuyasu (47). Specimens were examined in a Philips EM-300 electron microscope at 80 kV.

SDS-PAGE Immunoblotting Analysis of Sgp 130 and Vinculin Expression in Gizzard and Cardiac Membrane Nonjunctional and Junctional Isolates

Samples of a 10 mM CHAPS detergent gizzard homogenate, nonjunctional, and junctional cardiac membranes isolated on sucrose gradients (8, 34) were generally prepared for SDS-PAGE using mild sample buffer conditions (0.1–1.0% SDS final concentration, absence of reducing agents and heat), empirically discovered in a previous report (40) to preserve mAb 30B6 recognition of its antigen, i.e., the nonreduced, 107-kD form of Sgp 130. Molecular weight standards were prepared according to the methods of Lane (33). Solubilized samples were subjected to electrophoresis in 7.5% polyacrylamide slab gels by the method of Laemmli (32). Gels were stained with silver nitrate (35). For immunoblotting, gels were transferred to nitrocellulose sheets (Millipore Corp., Bedford, MA) in running buffer used for Laemmli-SDS-PAGE at 0.5 mA for 90 min. After transfer the replicas were rinsed three times for 10 min with 20 mM Tris HCl, 0.5 M NaCl, pH 7.4 and were blocked overnight at 4°C with 3% BSA in the same buffer.

For the gizzard and cardiac membrane samples, a separate replica was processed for detection of Sgp 130 and vinculin. Reaction of replicas was with either mAb 30B6 or affinity-purified rabbit anti-vinculin for 2 h at room temperature at 10 μg/ml in 20 mM Tris HCl, 0.5 M NaCl, 3% BSA, respectively, followed by (*a*) an affinity-purified rabbit anti-mouse IgG for 90 min at 37°C followed by an affinity-purified horseradish peroxidase conjugate of goat anti-rabbit IgG for 2 h at room temperature (Cooper Biochemical Inc.) (for detection of bound mAb 30B6), or (*b*) an affinity-purified horseradish peroxidase conjugate of goat anti-rabbit IgG for 2 h at room temperature (for detection of bound rabbit anti-vinculin antibodies). All blots were reacted with diaminobenzidine at 500 μg/ml in 50 mM Tris-HCl pH 7.5 with 0.03% hydrogen peroxide.

Results

Coincident and Differential Distributions of Sgp 130 and Vinculin in Cultured Fibroblasts and Endothelial Cells

Two cultured cell types with prominent bundled arrays of actin microfilaments (fibroblasts and endothelial cells) were selected for study of the relative distributions of Sgp 130 and vinculin by double indirect immunofluorescent detection. In these experiments fixed cells were treated with 0.1% Triton X-100 for 5 min before incubation with mAb 30B6 and rabbit anti-vinculin antibodies. In both chick embryonic fibroblasts (Fig. 1, *A* and *B*) and aortic endothelial cells (Fig. 1, *C–E*), Sgp 130 and vinculin were detected coincidentally in patch-like distributions at sites along the cell periphery that are known to correspond to the focal contact sites. A substantial amount of vinculin was detected throughout the cytoplasm of both cell types (Fig. 1, *B* and *E*); Sgp 130 was also observed at perinuclear sites (Fig. 1 *A*). A differential concentration of Sgp 130 relative to vinculin was observed at restricted regions along the cell periphery. At these leading-edge sites (Fig. 1 *C, arrowhead*) Sgp 130 was found uniformly concentrated (Fig. 1 *D, arrowhead*), whereas vinculin was detectable as small discrete spots along the membrane (Fig. 1 *E, arrowhead*).

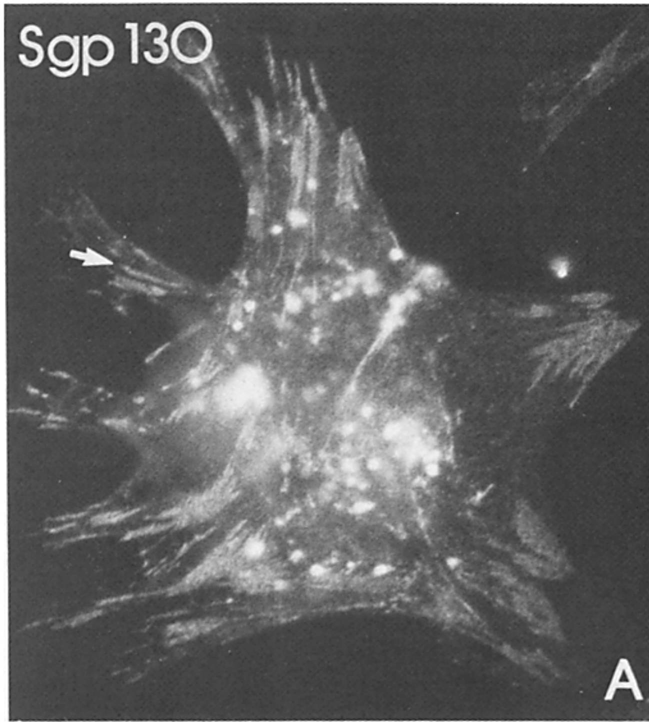
If fixed but nonpermeabilized fibroblasts and endothelial cells were mechanically disrupted with a stream of buffer, patches of ventral surface membrane remained attached to the substratum (Fig. 2, *A–F*). These membrane patches were coincident with dark interference reflection patterns (Fig. 2 *C, arrow*). In double indirect immunofluorescent experiments, a striking coincidence of the cell surface Sgp 130 (Fig. 2, *A* and *E*) and vinculin (Fig. 2, *B* and *F*) could be demonstrated at these membrane sites.

Sgp 130 and Vinculin Are Co-Concentrated in the Membrane Dense Plaque of Gizzard Smooth Muscle

Biochemical isolation studies have shown that Sgp 130 and vinculin are expressed at the most significant levels in gizzard smooth muscle (18, 40). As a compact, nearly homogeneous tissue abundant with structurally discrete microfilament-attachment sites to the plasma membrane (membrane dense plaque), gizzard smooth muscle would therefore be predicted as a favorable tissue type to study at high resolution the possible in situ interrelationships between Sgp 130 and vinculin.

In this study our immunolabeling approach allowed for the first time resolution of Sgp 130 at discrete sites along the bilayer. This was accomplished by using low concentrations of mAb 30B6 (3–10 μg/ml) and secondary detection with affinity-purified, biotinylated $F(Ab')_2$ fragments of goat anti-mouse IgG followed by either fluorescein- or ferritin-conjugated streptavidin. Semithin frozen sections of gizzard smooth muscle similarly immunolabeled with mAb 30B6 followed by rabbit anti-vinculin antibodies are shown in Fig. 3, *A–D*. Sgp 130 (Fig. 3, *A* and *B*) and vinculin (Fig. 3, *C* and *D*) are coincidentally concentrated at repeated sites along the plasma membrane. In these immunolabeled preparations vinculin often appears to precisely underscore the discontinuous appearance of Sgp 130 (Fig. 3, *A–D, arrows*).

Similar kinds of double immunolabel experiments were carried out with ultrathin frozen sections of gizzard smooth muscle. For these high resolution studies, ferritin-streptavidin was used to indirectly label bound mAb 30B6 to Sgp 130 via a biotinylated goat anti-mouse IgG, whereas an iron-dextran (Imposil) secondary conjugate was used to detect bound rabbit antibodies to vinculin. In heavily positive-stained frozen sections, the electron-dense membrane plaque of smooth muscle is revealed as the site where bundles of microfilaments terminate in the plasma membrane (Fig. 4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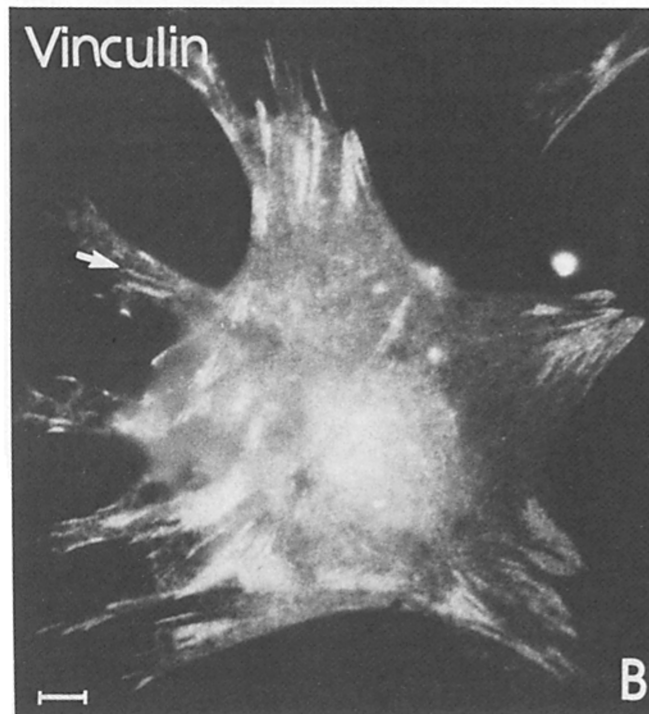
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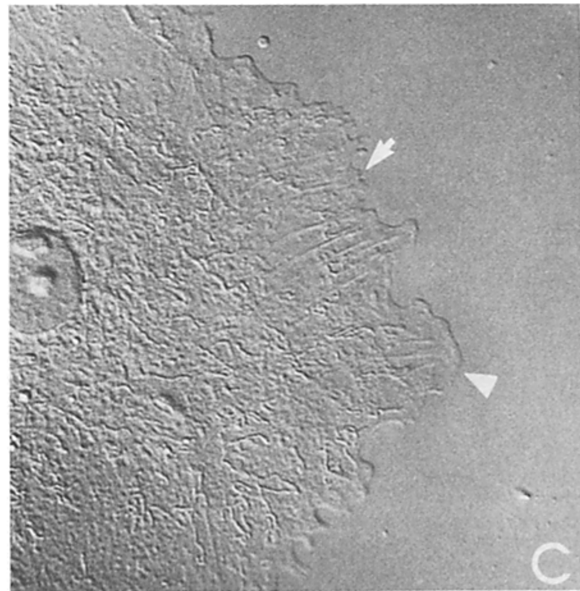
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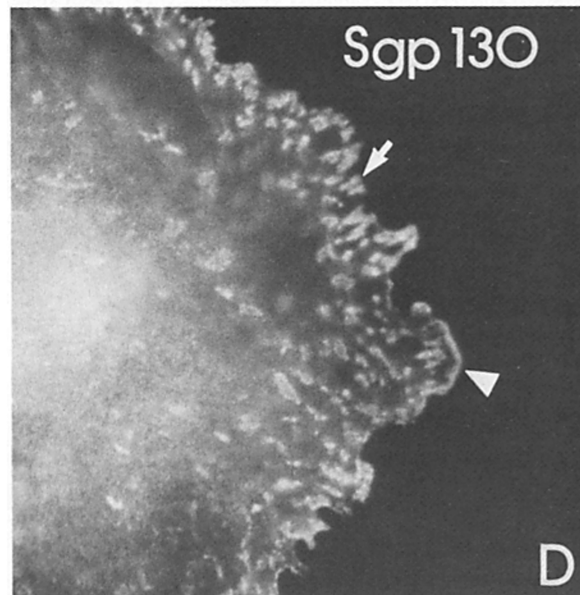
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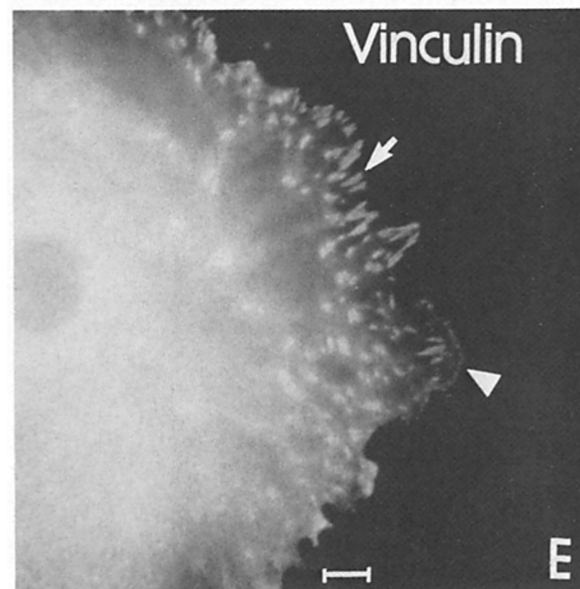
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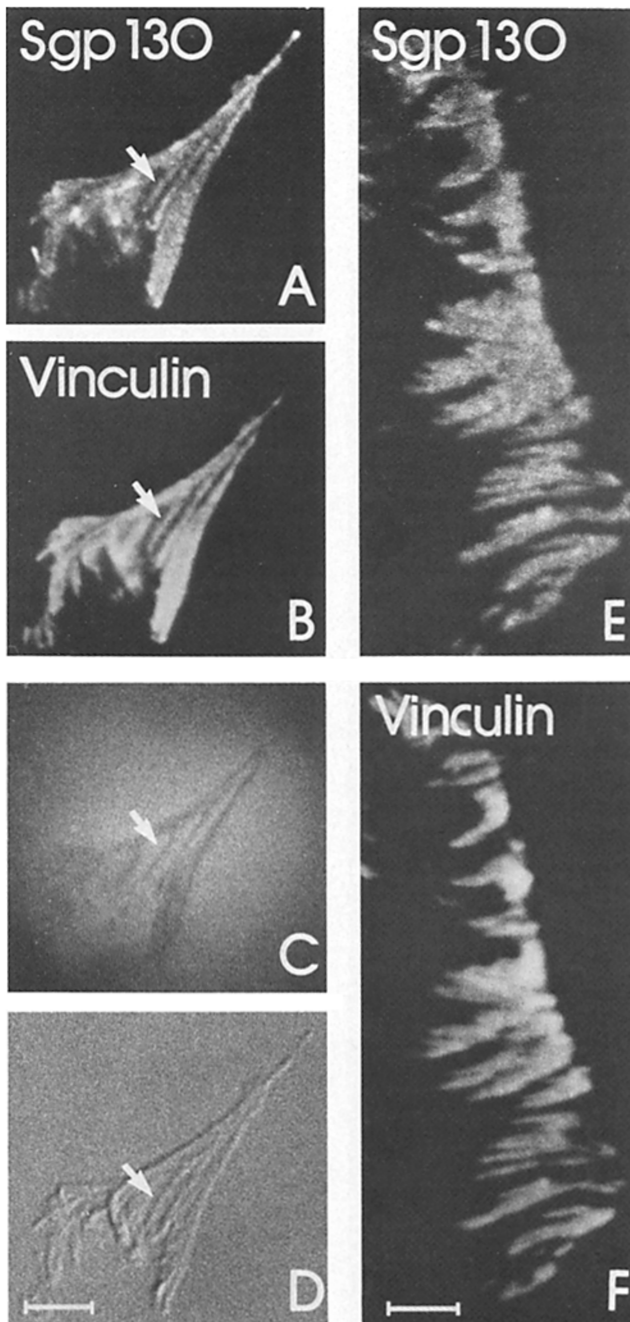


Figure 2. Peripheral sites of ventrally attached membranes from fixed, non-Triton X-100 treated, mechanically dislodged chicken embryo fibroblasts (*A–D*) and aortic endothelial cells (*E* and *F*). Double indirect immunofluorescent detection of the cell surface Sgp 130 species (*A* and *E*) and vinculin (*B* and *F*) shows a direct correspondence between these two membrane-associated proteins (*arrows*), as well as with the dark patterns in interference reflection microscopy (*C, arrow*), and the image seen in Nomarski optics (*D, arrow*). Bar, 10 μm.

A). In lightly positive-stained frozen sections (Fig. 4, *B–D*), these membrane dense plaques are immunolabeled for both vinculin (Imposil conjugate; *arrowhead, v*), and Sgp 130 (ferritin conjugate; *arrows, Sgp*). Vinculin is resolved along the electron-dense layer underlying the plasma membrane, whereas Sgp 130 is found directly above at the level of the membrane bilayer (Fig. 4, *B–D*). Along stretches of the cell membrane peripheral to the membrane-associated dense plaques (Fig. 4, *C* and *D, open arrows*), no significant concentrations of Sgp 130 or vinculin are observed. However, small microdomains with coincident concentrations of Sgp 130 and vinculin were occasionally detected (as shown in Fig. 4 *D*). Neither Sgp 130 nor vinculin were immunodetected at the intracellular dense bodies (data not shown).

Sgp 130 and Vinculin Are Co-Concentrated at Dense Plaque Sites of Myofibril Attachment to the Sarcolemma of Cardiac Muscle but Not in the Fascia Adherens Junctions

Double immunolabeling experiments were carried out with the ventricular papillary muscle of chicken hearts following the same general approach used for gizzard smooth muscle so as to simultaneously map the distributions of Sgp 130 and vinculin. In semithin sections both Sgp 130 (Fig. 5 *C*) and vinculin (Fig. 5 *B*) are coincidentally detected at periodic discrete sites along the sarcolemma (*arrows*). From the Nomarski differential contrast image (Fig. 5 *A, arrows*), these sites appear to be at the vicinity of Z band attachment to the sarcolemma. Vinculin is found exclusively in the intercalated disc region (Fig. 5 *B, ID, arrow*) where cardiac cells are known to be coupled end-on via a series of discrete junctional sites which include the vinculin-rich fascia adherens junctions (48). Sgp 130 was consistently not detectable, however, at the cardiac intercalated discs (Fig. 5 *C*).

To exclude the possibility of an accessibility problem for mAb 30B6 binding to Sgp 130 in the closely apposed junctional membranes of the intercalated region, two independent lines of experiments were pursued. The first series of experiments involved the structural uncoupling of cardiac cells at these junctional sites (8, 50) by perfusing unfixed, beating chicken hearts in a balanced salt solution containing 2 mM EGTA. In such EGTA-treated, fixed cardiac tissue, cells are often separated from each other along lateral margins, as well as at the intercalated disc sites. In double indirectly immunolabeled, ultrathin-sectioned specimens, Sgp 130 (ferritin-conjugate; *arrows, Sgp*) remained immunologically undetectable along the exposed membrane (*open arrows*) of the fascia adherens sites (Fig. 6 *B*), whereas vinculin, represented by Imposil particles (*arrowhead, v*), was specifically detected within the electron-dense layer (Fig. 6, *A* and *B*). On the other hand, both Sgp 130 (*arrow*) and vinculin (*arrowhead*) (Fig. 6 *D*) were resolved at the region of Z band attachment to the sarcolemma (7) (Fig. 6, *C* and *D*) consistent with the previously shown semithin section results (Fig. 5, *A–C*).

Figure 1. Double indirect immunofluorescent staining of fixed and 0.1% Triton X-100 permeabilized chick embryo fibroblasts (*A* and *B*) and aortic endothelial cells (*C–E*) for the cell surface Sgp 130 species using mAb 30B6 (*A* and *D*) and affinity-purified rabbit anti-vinculin antibodies (*B* and *E*). A striking co-distribution of these two proteins is shown at the peripheral regions of both cell types (*arrows*). Significant levels of diffusely distributed vinculin are evident in *B* and *E*, whereas a discrete perinuclear distribution, probably Golgi-associated, is evident for Sgp 130 in *A* and *D*. Note the discrete spot-like distribution of vinculin relative to the uniform appearance of Sgp 130 along the leading edge of the endothelial cell (*C–E, arrowheads*). Bar, 10 μm.

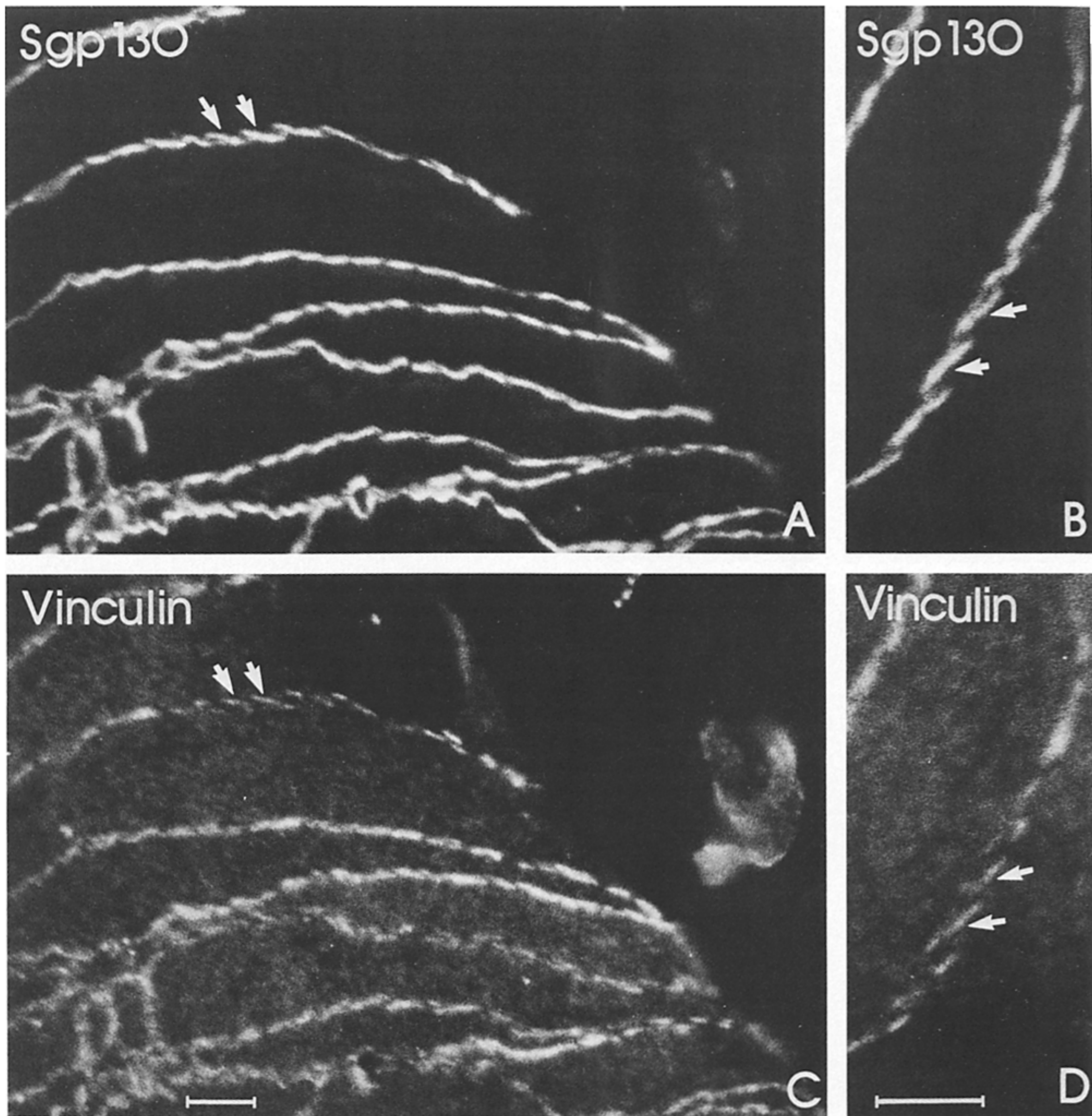
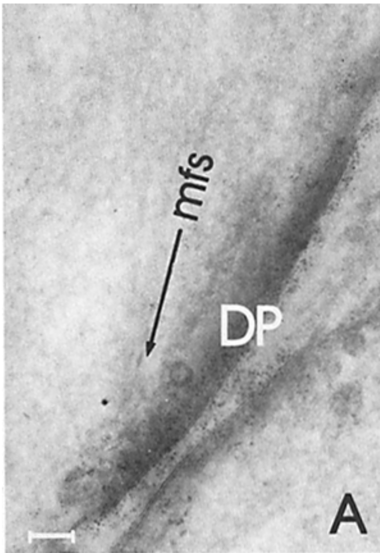


Figure 3. Double indirect immunofluorescent labeling of Sgp 130 (*A* and *B*) and vinculin (*C* and *D*) on semithin (0.5 μm) sections of fixed gizzard smooth muscle. Sgp 130 and vinculin are found in coincident arrays along the membrane (*arrows*), probably the membrane-associated dense plaque sites. *B* and *D*, respectively, represent a magnified portion of *A* and *C* (*arrows*), and demonstrate the prominent, repeating nature of the immunofluorescent arrays. Bar, 10 μm.

Figure 4. Resolution of vinculin (Imposil label; *arrowhead, v*) and Sgp 130 (ferritin label; *arrow, Sgp*) at the membrane-associated dense plaque (*DP*) of gizzard smooth muscle by double indirect immunoelectron microscopy. In heavily positive-stained ultrathin sectioned tissue (*A*) the membrane dense plaque is shown to be a prominent site of microfilament–membrane attachment. *B–D* are representative double indirect immunoelectron microscopic labeled, ultrathin frozen sections using ferritin-conjugated streptavidin to detect bound biotinylated rabbit anti–mouse IgG to the primary antibody mAb 30B6 (*arrow,* Sgp 130), and iron-dextran (Imposil) conjugated goat anti–rabbit IgG to detect bound rabbit antibodies to vinculin (*arrowhead, v*). Vinculin and Sgp 130 are often found coincidentally concentrated at the membrane dense plaques with vinculin found along the cytoplasmic electron dense material and Sgp 130 directly above at the membrane bilayer. Membrane sites peripheral to the membrane dense plaque (*C* and *D, open arrows*) have low labeling for vinculin as well as Sgp 130. Discrete microdomains of vinculin and Sgp 130 are occasionally observed outside the prominent electron dense plaque regions (*D*). Bar, 0.1 μm.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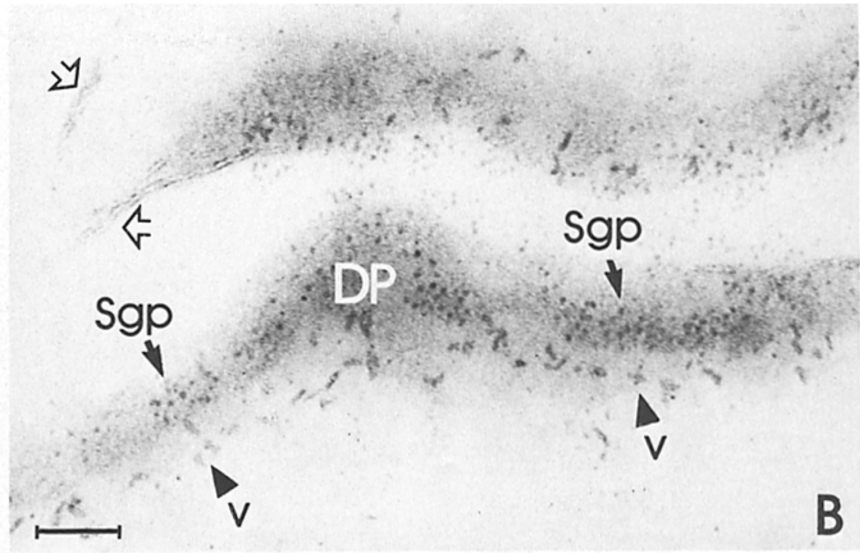
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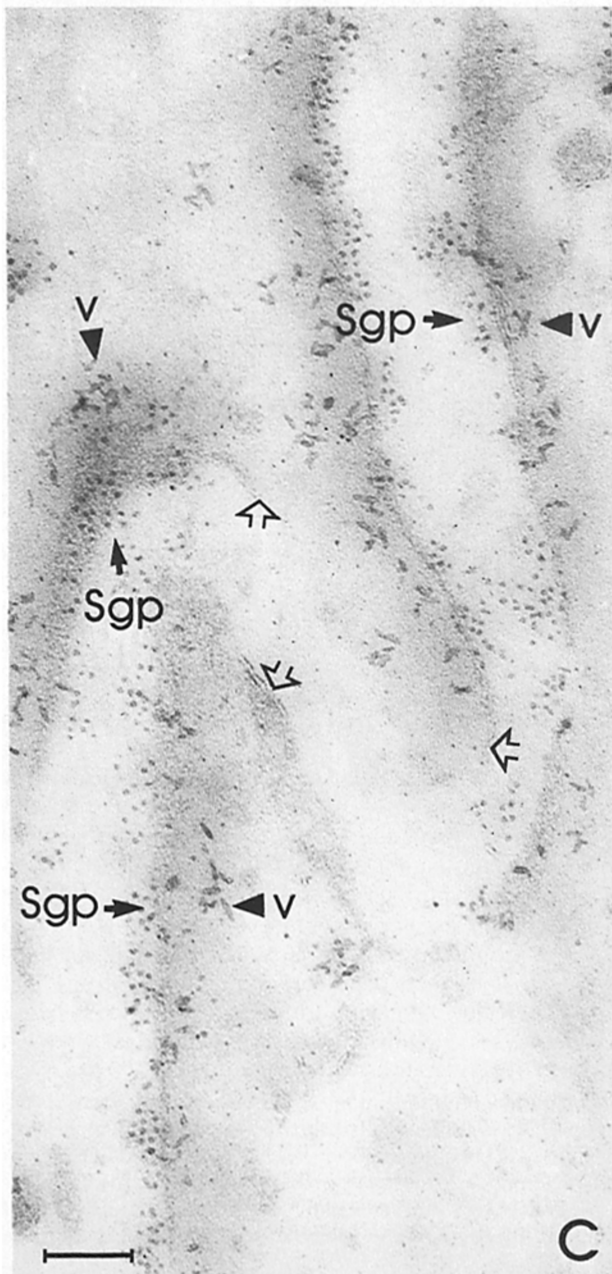
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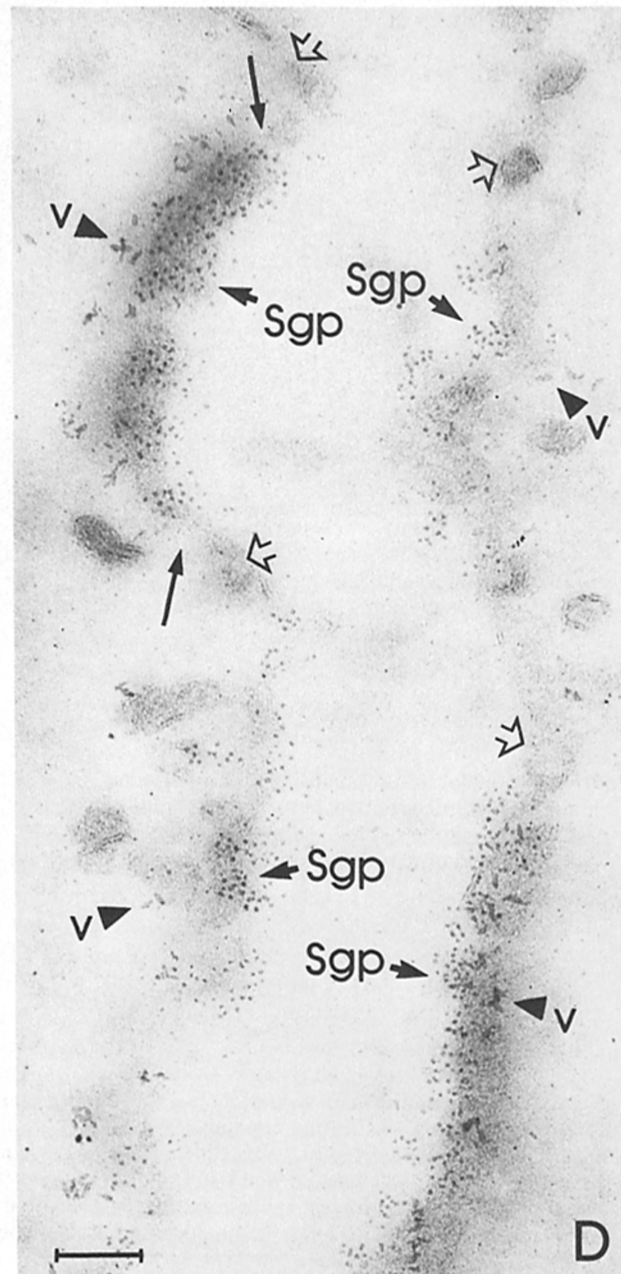
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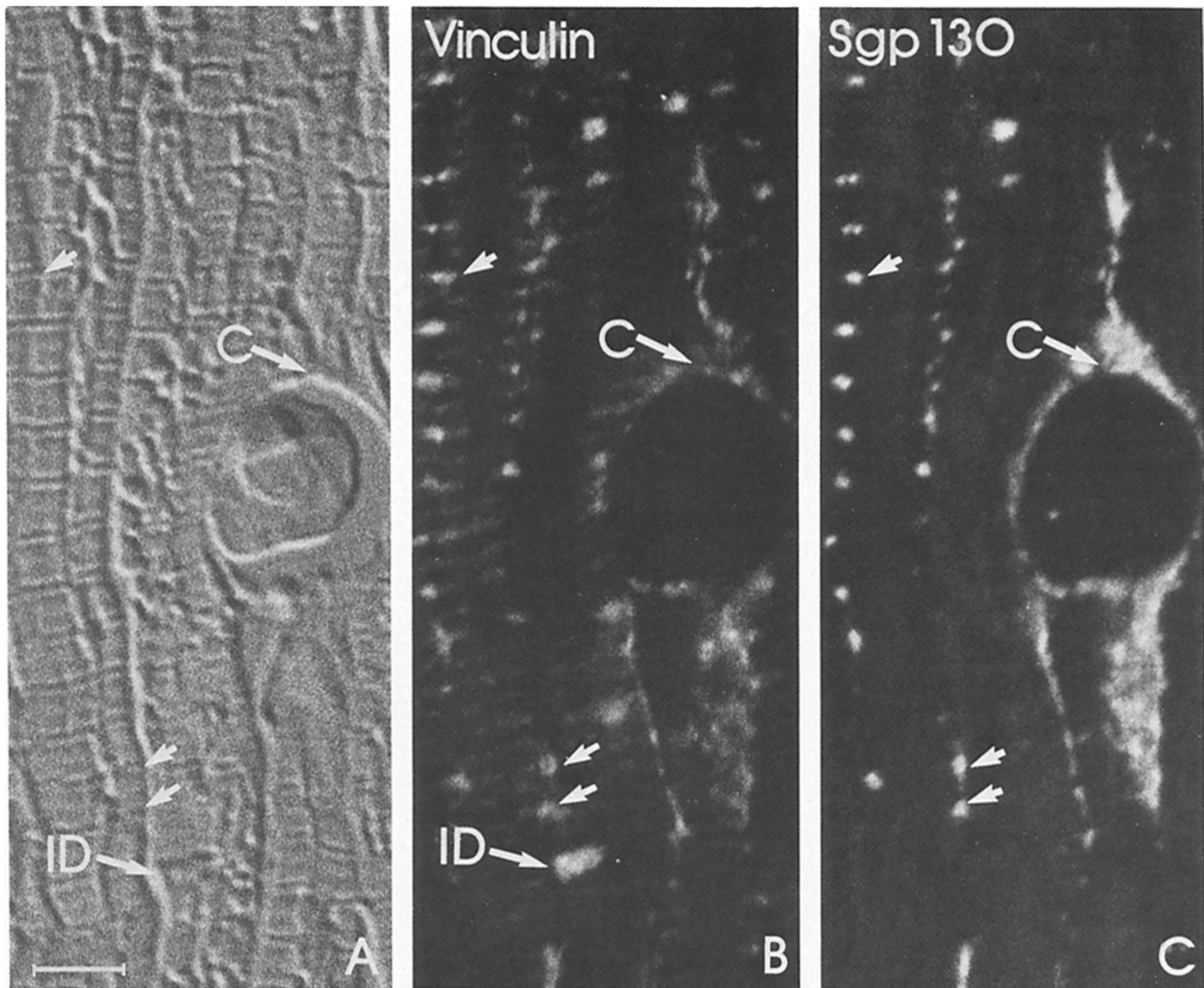
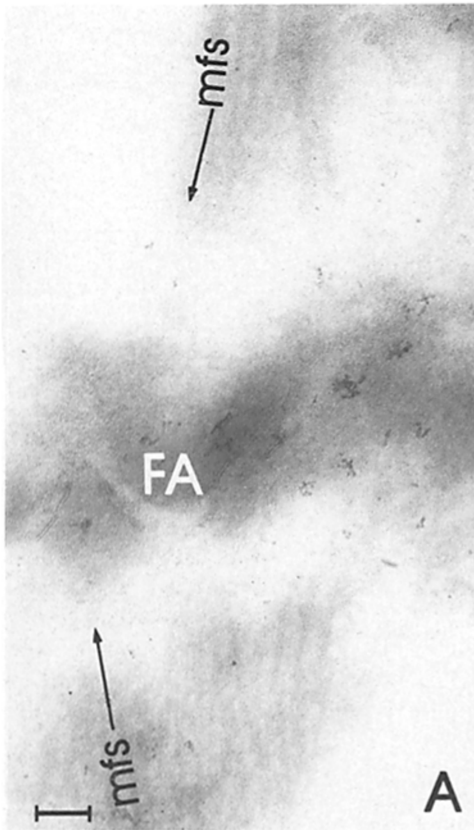
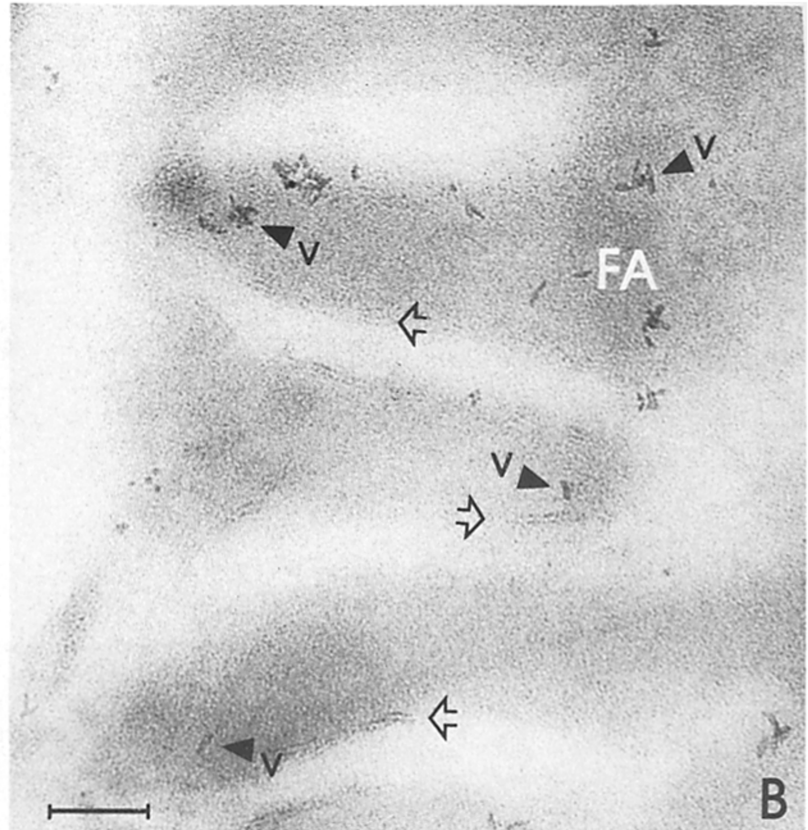


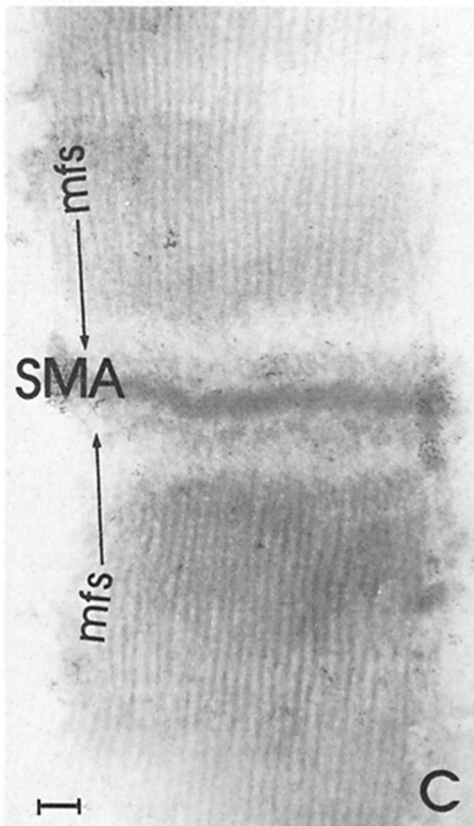
Figure 5. Double indirect immunolabeling for Sgp 130 and vinculin in semithin sections of fixed cardiac tissue. Note the regular coincidence of the Sgp 130 species (*C*) and vinculin (*B*) along the plasma membrane (sarcolemma; *arrows* in *A*), but not at the intercalated disc sites (*ID*) where only vinculin is concentrated. A portion of a capillary (*C*) is also evident in this section. The Nomarski optics image is presented in *A*. Similar immunolabeling patterns are observed with EGTA-uncoupled cardiac tissue. Bar, 10 μm.

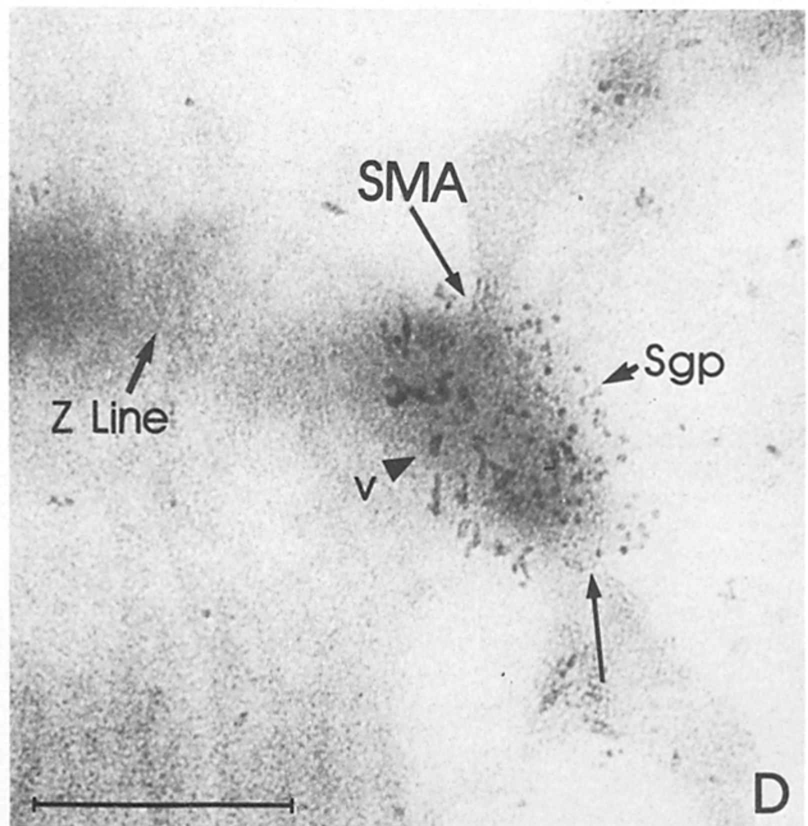
In a second series of experiments presented in Fig. 7, expression levels of Sgp 130 and vinculin were assessed by parallel double immunoblotting analysis using mAb 30B6 (lanes *a'*–*d'*) and affinity-purified rabbit anti–vinculin antibodies (lanes *a''*–*d''*) against protein equivalent amounts of 10 mM CHAPS gizzard extracts (lane *a*), crude, predominantly nonjunctional (lane *b*), crude intercalated disc junctional (lane *c*), and fascia adherens junctional-enriched (lane *d*) cardiac membranes under nonreducing conditons of SDS-PAGE. Under these nonreducing conditions the mAb 30B6 species (Sgp 130) assumes a molecular mass form of ∼107 kD rather than 130 kD. A mAb 30B6 species is detectable in the 10 mM CHAPS gizzard extract (lane *a'*), nonjunctional (lane *b'*) and crude junctional (lane *c'*), but not in the fascia adherens-rich fraction (lane *d'*). In the gizzard extracts (lane *a''*), vinculin and metavinculin (a 152-kD species [12]) are recognized by the affinity-purified rabbit antibody. Although barely detectable in nonjunctional (lane *b''*) and partially purified intercalated disc–containing junctional (lane *c''*) cardiac membranes, vinculin was found to be significantly enriched in the fascia adherens–containing junctional fraction (lane *d''*). Major trends in the double immunolabeling results are presented (Table I) and correlated with either nonjunctional, contractile-related force coupling

Figure 6. Double indirect immunodetection of Sgp 130 and vinculin on ultrathin frozen sections of EGTA-uncoupled, fixed cardiac tissue. Perfusion of excised, beating hearts with EGTA before fixation promoted separation of cells laterally and end-on at the intercalated disc sites (*A* and *B*). Despite increased accessibility of immunolabeling reagents in these preparations to the exposed fascia adherens junctional plasma membranes (sites where bundles of microfilaments [*mfs*, *arrows*] terminate in the membrane as shown in *A*), only vinculin is detectable (Imposil label; *arrowhead, v*). No significant detection of Sgp 130 is observed at the membrane bilayer (*B*, *open arrow*), although the experimental immunolabeling conditions were identical to those used for gizzard smooth muscle (Figs. 3 and 4). In the same cardiac

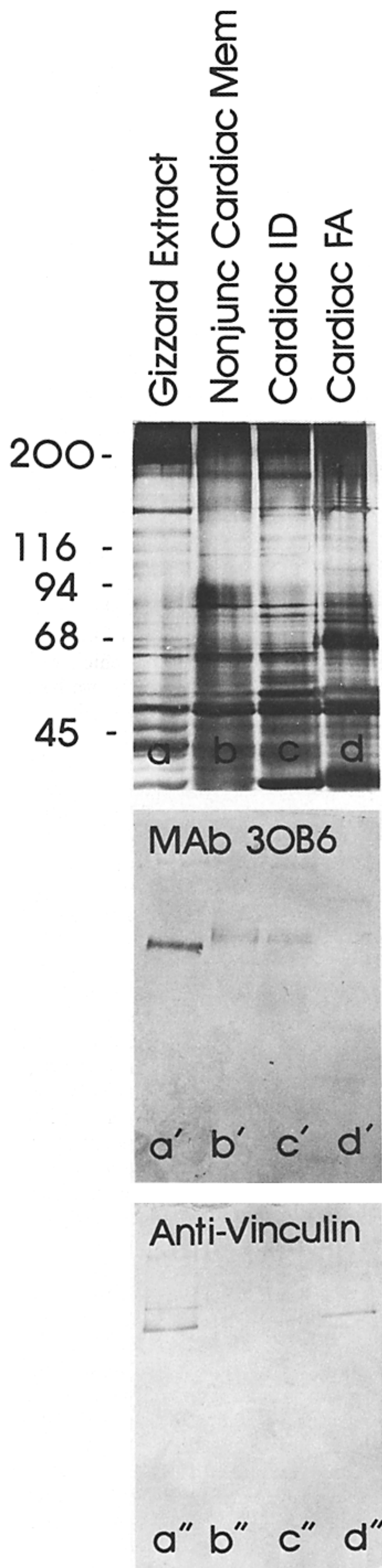








tissue preparations, double indirect immunolabel detection of Sgp 130 and vinculin at the sarcolemma–myofibril attachment sites (*SMA*) is facilitated as shown in *C* and *D*. Vinculin (Imposil label; *arrowhead, v*) is resolved at the membrane-associated dense plaque which overlies the Z line of the SMA (*D*), whereas Sgp 130 (ferritin label; *arrow, Sgp*) is restricted to a portion of the membrane directly above the sarcolemma-associated dense plaque. Bar, 0.1 μm.



sites (membrane-associated dense plaque of smooth and cardiac muscle) or junctional-related, cell-cell coupling (fascia adherens of cardiac muscle) microfilament-membrane attachment sites.

Discussion

In a series of double labeling studies using immunofluorescent, immunoelectron microscopic, and immunoblotting approaches, new evidence is provided for differential levels of Sgp 130 at plasma membrane sites cytoplasmically underlaid by vinculin. Sgp 130 appears to be preferentially associated at nonjunctional dense plaque sites with a contractile or force coupling function. We hypothesize that Sgp 130 has a major role in the molecular and functional organization of these dense plaque sites and that its distributions are stabilized in part by transmembrane linkages with a special subclass of vinculin molecules.

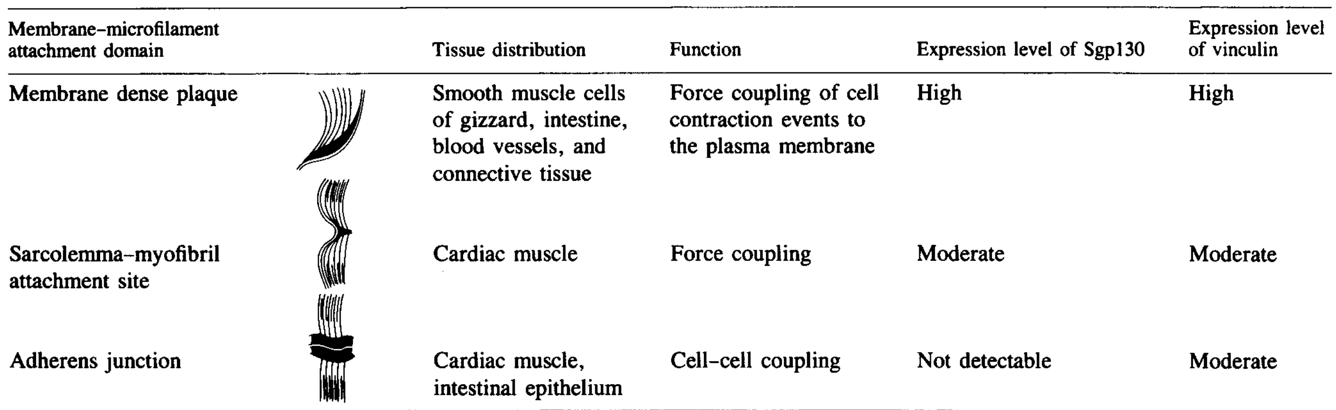
In a previous report (40) work was presented on the development of mouse mAb 30B6 to a membrane protein whose surface topologies are closely correlated with microfilament distributions in cultured interphase and cleaving mitotic cells. From immunoblotting, immunoaffinity chromatography, and immunoprecipitation experiments, it was concluded that the antigen of mAb 30B6 (now referred to as Sgp 130) is an integral sialoglycoprotein with a molecular mass of ∼130 kD under reducing conditions of SDS -PAGE. In adult chicken gizzard smooth muscle tissue, Sgp 130 may also interact with an immunologically unrelated 175-kD glycoprotein (Rogalski, A. A., manuscript in preparation). In a separate biochemical report to follow, we will also present detailed characterization of several tissue-specific Sgp 130 isoforms. The CSAT fibronectin receptor complex (26) may have secondary associations with one of the Sgp 130 isoforms. This is suggested, in part, because combined mAb 30B6 immunoblotting and GELCODE silver staining analysis of the three CSAT glycoproteins separated by SDS-PAGE shows Sgp 130 in trace amounts as a minor red band electrophoretically overlapping the position of a brown, broadly resolved CSAT band 3. When mAb 30B6-immunoprecipitated Sgp 130 from eviscerated embryo extracts is similarly analyzed, it appears as a homogeneous red band.

Nature of Sgp 130 In Vivo Distributions Relative to Vinculin

Previous immunoelectron microscopic observations (Geiger et al. [18]; Tokuyasu et al. [48]; Chen and Singer [5]) have

Figure 7. Double immunoblotting analyses with the mAb 30B6 probe for Sgp 130 (*a′*–*d′*) and rabbit anti-vinculin (*a″*–*d″*) of SDS-PAGE resolved polypeptides of gizzard smooth muscle 10 mM CHAPS detergent extracts (lane *a*), crude, predominantly nonjunctional cardiac membranes (lane *b*), crude intercalated disc (*ID*) cardiac membranes (lane *c*), and fascia adherens-rich (*FA*) fractions (lane *d*). Lanes *a*–*d* of this silver nitrate-stained gel represent identical loads of SDS-solubilized peptides under nonreducing conditions. A mAb 30B6-107 kD specific species (nonreduced form of Sgp 130) and vinculin (including the immunologically related, 152-kD metavinculin) are both detectable only in soluble 10 mM CHAPS extracts of gizzard smooth muscle. Note the absence of the mAb 30B6 species in the adherens junction fraction, a result consistent with the in vivo immunolabeling tissue data.

Table I. Distribution Patterns for Sgp130 and Vinculin



Membrane–microfilament attachment domain	Tissue distribution	Function	Expression level of Sgp130	Expression level of vinculin
Membrane dense plaque	Smooth muscle cells of gizzard, intestine, blood vessels, and connective tissue	Force coupling of cell contraction events to the plasma membrane	High	High
Sarcolemma–myofibril attachment site	Cardiac muscle	Force coupling	Moderate	Moderate
Adherens junction	Cardiac muscle, intestinal epithelium	Cell–cell coupling	Not detectable	Moderate

An immunolabeling summary table illustrating the major distribution patterns for both Sgp 130 and vinculin in selected tissues abundant in structurally prominent microfilament–membrane attachment sites, i.e., gizzard smooth muscle with the membrane dense plaque and cardiac muscle with the dense plaque associated sarcolemma–myofibril attachment sites and fascia adherens junctions. Note the general trend for the association of Sgp 130 with the nonjunctional membrane-associated dense plaque sites where cytoskeleton force-generating or contractile activities are coupled to the plasma membrane, but the absence of this membrane species from adherens-junctional, cell–cell coupling sites.

shown that the relative in vivo spatial arrangements of the cytoskeletal proteins, alpha-actinin, tropomyosin, and vinculin, were well conserved in cultured cells and tissues at structurally and functionally distinct microfilament attachment sites. For instance, at the membrane-dense plaque of smooth muscle and fascia adherens junctions of cardiac muscle, vinculin was consistently situated at a closer proximity to the plasma membrane than alpha-actinin, whereas tropomyosin was excluded from these sites and diffusely found in the immediate adjacent cellular regions. From these investigations, it appears that certain cytoskeletal components are widespread in their association with microfilament–membrane attachment sites.

In this study, Sgp 130 was shown for the first time to have site-specific variation in its distributions relative to vinculin. One of the most striking examples of this phenomenon involved the nonjunctional membrane dense plaque of gizzard smooth muscle (where Sgp 130 and vinculin are coincidentally concentrated) and the junctional fascia adherens membrane of cardiac muscle (where Sgp 130 is immunologically undetectable) both in the in vivo (intact and EGTA-uncoupled intercalated discs) and in vitro, isolated forms even though vinculin is sharply confined and strongly associated (resistant to Sarkosyl extraction) at these junctional sites. An additional observation in line with the cardiac adherens junction immunolabeling result is that Sgp 130 is not detectable at the zonula adherens of the intestinal brush border epithelial cells (unpublished data), whereas vinculin is sharply concentrated at these sites (18). Since a 30B6-specific species was undetectable in immunoblotting assays of SDS-PAGE resolved fascia adherens, it argues against the possibility that in vivo the 30B6-related epitope is masked by other proteins or is inaccessible to antibody labeling reagents. Another example of variation in Sgp 130 distributions involves the fascia adherens and focal contacts of cultured cells. It has been suggested that these cell membrane adhesion structures are molecularly related (34, 44, 48). For instance, cytoskeletal proteins, such as vinculin (5, 48) and a 200-kD protein (34), are concentrated at both the fascia adherens and focal contacts. Our finding here that Sgp 130 is associated with the focal contacts of cultured embryonic cells, but not the fascia adherens of adult cardiac muscle, clearly demonstrates, on the other hand, that these microfilament–membrane attachment structures are distinguishable at the molecular level of integral protein components. Since Sgp 130 has widespread actin-related surface associations in cultured chick embryonic cells, including intercellular contact sites, we have not yet ruled out the possibility that it is associated with immature or developing forms of the fascia adherens.

One attractive explanation for the segregation of Sgp 130 to nonjunctional, force coupling sites, such as the membrane dense plaque, cleavage furrow of dividing cells, or leading edge of interphase cells, is that this membrane species participates in functions primarily related to coupling the plasma membrane to the underlying contractile cytoskeleton rather than cell–cell coupling. This contractility function may not be necessarily dependent on the peripheral protein vinculin, or alternatively may involve a dynamic relationship between Sgp 130 and vinculin with Sgp 130 assuming a more primary role. Some aspects of this hypothesis would be consistent with observations that vinculin is not concentrated in the cleavage furrow of dividing cells (20) where contractile ring microfilaments are known to be intimately associated with the cytoplasmic side of cell membrane (43). As a transient microfilament–membrane domain, the cleavage furrow is very likely too dynamic in nature for stable vinculin associations to occur in the form of dense plaques. Also, along the lines of this hypothesis, our finding for a concentration of Sgp 130 at the leading, active edge and focal contacts of cultured cells implies that these membrane sites are either heterogeneous in molecular composition or interchangeable in cellular functions of contractility and adhesion (28, 39, 49).

Other explanations have not been ruled out for the distribution of Sgp 130. One could be based on structural rather than functional criteria, such as the nature of the approach of microfilaments to the plasma membrane (end-on as opposed to lateral). However, such a hypothesis would be dependent on and complicated by limited structural evidence especially since the exact nature of microfilament insertion into the plasma membrane of many of these attachment sites is obscured by a prominent electron-dense plaque.

From earlier mAb 30B6 immunoblotting surveys (40) and

subsequent unpublished work with various tissue and eviscerated embryo detergent extracts, Sgp 130 has been found to have its highest expression levels in gizzard smooth muscle, a dense, nearly homogeneous tissue with a predominantly contractile function (16). The present study now provides a basis for these observations because Sgp 130 is highly concentrated in the membrane-associated dense plaque region, and prominent and highly abundant microfilament attachment sites. The technology of double indirect immunoelectron microscopy with the preferred attachment topologies of ferritin antibody conjugates (30-nm resolution) and iron-dextran (Imposil) antibody conjugates (35–40 nm resolution) (43) has permitted detection of Sgp 130 at the membrane bilayer of the dense plaque region with vinculin immediately below in the cytoplasmic electron dense layer. From this new immunolabeling data it would appear that the epitope on Sgp 130 (recognized by mAb 30B6) is restricted to the external side of the plasma membrane. It does not, however, rule out the possibility that the Sgp 130 molecule traverses the membrane bilayer and extends to the cytoplasmic side of the membrane.

Nonjunctional Dense Plaques as a Distinct Class of Plasma Membrane Structures

In this report indirect double immunoelectron microscopy has permitted simultaneous resolution of Sgp 130 and vinculin at the membrane dense plaque of gizzard smooth muscle and sarcolemma dense plaque of cardiac muscle, but not the fascia adherens junctions. To our knowledge this is the first demonstration of a transmembrane-like co-concentration of a known integral and peripheral protein at nonjunctional dense plaque sites. The work of Geiger et al. (18) and Pardo et al. (37) have, respectively, provided the first lines of evidence for vinculin concentration at smooth muscle membrane dense plaques and cardiac muscle Z line attachment plaques to the sarcolemma (costameres). In contrast, the CSAT/JG22 fibronectin receptor glycoproteins (24, 26) have been reported to be restricted to peripheral regions of the membrane dense plaque of gizzard smooth muscle (6) quite similar to a predominantly peripheral distribution at focal contact sites (11). The peripheral distribution of extracellular matrix receptors implies that these molecules have a secondary involvement in the molecular organization of dense plaque structures.

Our immunolabeling results persuade us to tentatively place smooth and cardiac muscle membrane-associated dense plaque sites in a molecularly and functionally distinct class of membrane structures rather than a subgroup of adherens junctions, thus modifying the original classifications of Geiger et al. (21). Both of the dense plaque structures probably have primary roles not in cell–cell coupling, but instead in the mechanical coupling or stabilization of the plasma membrane to the underlying contractile cytoskeleton. We would also include the cleavage furrow of dividing cells in the same category as nonjunctional membrane dense plaques even though the cleavage furrow lacks a cytoplasmic dense plaque structural component. As discussed previously, the developmentally transient force coupling activities of the cleavage furrow probably antagonize formation of stable dense plaques and concentration of peripheral proteins like vinculin.

By two independent immunolabeling approaches we showed that vinculin, but not Sgp 130, is associated with the fascia adherens junctional domains of cardiac muscle. This finding has important structural and functional implications for vinculin and Sgp 130. At adherens junctions vinculin appears to have an unusually high-affinity association. This is evident because it is retained in isolated junctional fractions derived from Sarkosyl detergent extraction of cardiac muscle, an atypical behavior for a peripheral membrane protein. A unique vinculin isoform (19) with remarkably stable membrane associations, therefore, might be expressed at these adherens sites. In contrast, our finding that Sgp 130 is not associated with the fascia adherens strongly implies that this membrane species is functionally unrelated to stable cell–cell coupling junctions and its associated vinculin isoforms. That other integral proteins have this functional specialization would seem to be supported by recent evidence (50) for a 135-kD membrane protein that is indeed adherens junction–specific but not detectable in the membrane dense plaque of smooth muscle.

Taken together our observations in this report suggest that Sgp 130 may assume a more primary and dynamic role in the plasma membrane organization of microfilament attachment sites and cell contractility functions than previously suspected (40). This is a possibility that we are now investigating.

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