

# Localization of a Heterotrimeric G Protein $\gamma$ Subunit to Focal Adhesions and Associated Stress Fibers

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**Abstract.** Signal transducing heterotrimeric G proteins are responsible for coupling a large number of cell surface receptors to the appropriate effector(s). Of the three subunits, 16  $\alpha$ , 4  $\beta$ , and 5  $\gamma$  subunits have been characterized, indicating a potential for over 300 unique combinations of heterotrimeric G proteins. To begin deciphering the unique G protein combinations that couple specific receptors with effectors, we examined the subcellular localization of the  $\gamma$  subunits. Using anti-peptide antibodies specific for each of the known  $\gamma$  subunits, neonatal cardiac fibroblasts were screened by standard immunocytochemistry. The anti- $\gamma_5$  subunit antibody yielded a highly distinctive pattern of intensely fluorescent regions near the periphery of

the cell that tended to protrude into the cell in a fibrous pattern. Dual staining with anti-vinculin antibody showed co-localization of the  $\gamma_5$  subunit with vinculin. In addition, the  $\gamma_5$  subunit staining extended a short distance out from the vinculin pattern along the protruding stress fiber, as revealed by double staining with phalloidin. These data indicated that the  $\gamma_5$  subunit was localized to areas of focal adhesion. Dual staining of rat aortic smooth muscle cells and Schwann cells also indicated co-localization of the  $\gamma_5$  subunit and vinculin, suggesting that the association of the  $\gamma_5$  subunit with areas of focal adhesion was widespread.

**H**ETEROTRIMERIC G proteins are responsible for transmitting signals from a large variety of ligand-bound receptors to specific effector(s), which, in turn, modulate the concentrations of intracellular signals, including cyclic-AMP, inositol 1,4,5-trisphosphate, diacylglycerol, and ions such as  $K^+$  and  $Ca^{2+}$  (reviewed in references 15, 39). Given its interposition between receptor and effector, much of the fidelity of signal transmission must reside in the  $\alpha\beta\gamma$  subunit structure of the G protein. While it has generally been assumed that structural heterogeneity of the  $\alpha$  subunits provides the specificity for receptor-G protein and G protein-effector interactions (reviewed in reference 46), a growing body of evidence indicates that a similar structural heterogeneity of the  $\beta$  and  $\gamma$  subunits exists, and that this heterogeneity, particularly in the  $\gamma$  subunits, contributes to the specificities of these interactions (24, 25, 35, 37, 45). While the functional significance of the individual  $\beta$  and  $\gamma$  subunits identified thus far has yet to be elucidated, evidence indicates that  $\beta\gamma$  subunits directly regulate the activities of several effectors. The rapidly expanding family of  $\beta\gamma$  regulated effectors include the type II and IV adenylyl cyclase (11, 22, 48), phospholipase  $A_2$  (23), the  $\beta$  family of phospholipase (3, 6, 47), muscarinic and  $\beta$ -adrenergic receptor kinases (16, 36), and a plasma membrane  $Ca^{2+}$

pump (27). In addition, evidence indicates that  $\beta\gamma$  subunits directly interact with receptors and contribute to the specificity of G protein-receptor interaction (34). Hence, receptor activation of heterotrimeric G protein produces a bifurcating signal that results in effector regulation by both the  $\alpha$  subunits and the  $\beta\gamma$  subunits (2, 9, 46).

Presently, there is little information regarding which combinations of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits define specific G proteins that participate in particular receptor-effector signaling pathways. Five  $\gamma$  subunits have been identified at the molecular and protein level. These include  $\gamma_1$ , which is found exclusively in retina (19),  $\gamma_2$  (43), and  $\gamma_3$  (14), which are found predominantly in brain, and the more recently described  $\gamma_5$  (12) and  $\gamma_7$  (5), which are widely distributed in brain and peripheral tissues. In studies examining highly specialized tissues that express a limited number of G protein subunits, it has been possible to identify the subunit composition of G protein-coupled pathways by immunocytochemical localization. For example, in monkey retina, the  $\alpha_1$  subunit of transducin ( $G_t$ ) is expressed exclusively in the rod outer segments in the photoreceptor layer of the retina, whereas the  $\alpha_2$  subunit of  $G_t$  is expressed exclusively in the cone outer segments (26). Examination of the  $\gamma$  subunits in this tissue revealed that only the  $\gamma_1$  subunit is expressed in the rod outer segments, while  $\gamma_2$ ,  $\gamma_5$ , and an unidentified  $\gamma$  subunit are expressed in the cone outer segments (33). Hence, these data suggests that the  $\gamma_1$  subunit associates with the  $\alpha_1$  subunit of  $G_t$ , whereas the  $\gamma_2$  and  $\gamma_5$  subunits

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possibly associate with the  $\alpha_2$  subunit of  $G_i$ . Using a similar approach, we examined whether specific G protein  $\gamma$  subunits could be localized to particular cell types and structures within less specialized tissues. A variety of cell types were immunostained with anti-peptide antibodies specific to each of the known  $\gamma$  subunits (5, 42). In the case of  $\gamma_5$ , a highly distinctive, immunostaining pattern was revealed. This pattern showed complete overlap with vinculin immunostaining and, to some extent, with phalloidin staining along the actin filaments. The  $\gamma_5$  immunostaining was extractable with a low Triton X-100 concentration, which did not affect either vinculin or actin staining. These data indicated that the  $\gamma_5$  subunit was localized to membranes associated with sites of focal adhesions. The localization of G protein to regions of focal adhesion strengthens the emerging role for focal adhesions as sites where G protein- and extracellular matrix-dependent pathways converge to regulate both cell adhesion and cell growth.

## Materials and Methods

### Cell Culture

Neonatal cardiac fibroblasts from 1-3-d-old Sprague-Dawley rat pups were prepared and cultured as previously described (13). Freshly isolated cells were grown in MEM containing MEM vitamins, MEM amino acids, 7.5% newborn calf serum, and penicillin-streptomycin. After 3 d, the cells were split (1:3) following trypsinization, and plated onto either 18 mm glass coverslips or onto 150 mm dishes. Cells were used for immunofluorescence or protein analysis 3 d later. Schwann cells were isolated, cultured, and transfected with the proteoglycan syndecan-1, as previously described (7, 8). Rat aortic smooth muscle cells were prepared as previously described (17).

### Anti- $\gamma$ Subunit Antibodies

All antisera directed against G protein  $\gamma$  subunits have been previously described and characterized (5, 42). Briefly, peptide sequences unique to each of the known  $\gamma$  subunits were synthesized, coupled to keyhole limpet hemocyanin, and injected into rabbits. Antisera were characterized for their specificity by immunoblotting extracts of baculovirus-infected Sf9 cells expressing each of the known  $\gamma$  subunits, as previously described (22, 40). Anti- $\gamma_5$  subunit antisera used in this study were obtained from two different rabbits, designated D-9 and D-10, that were injected with the peptide corresponding to amino acid residues 50-63 of  $\gamma_5$ . In addition, a portion of the D-10 antisera was purified by affinity chromatography on  $\gamma_5$  peptide-coupled Sepharose. All anti- $\gamma_5$  subunit antisera yielded the same immunostaining pattern. All data shown are with the D-10 antisera.

### Immunocytochemistry

Immunocytochemical localization of cellular protein was performed using standard techniques, as described previously (7). In general, cells were washed three times with PBS, fixed for 20 min in 4% paraformaldehyde in PBS, and subsequently permeabilized with 0.05% Triton X-100 for 5 min. The cells were blocked with 5% milk in TBS for 1 h, incubated with primary antibody in 5% horse serum for 1 h at 37°C, washed in TBS, and then incubated with the appropriate secondary antibody in 5% horse serum for 1 h at 37°C. Antisera were used at 1:200 dilution for anti- $\gamma$  subunit antibodies and 1:400 dilution for the anti-vinculin antibody. Phalloidin was used according to the manufacturer's instructions. Secondary antibodies used were FITC-conjugated goat anti-rabbit IgG (1:160) and TRITC-conjugated goat anti-mouse IgG (1:100).

### Immunoblotting

Neonatal cardiac fibroblasts grown on 150 mm culture dishes were washed three times and collected by scraping in 3 ml of 20 mM Hepes, 2 mM  $MgCl_2$ , and 1 mM EDTA, pH 7.2, containing 1 mM benzamide, and 10  $\mu$ g/ml each of leupeptin and pepstatin A (HME-PI). The cells were passed through a 25-gauge needle seven times and centrifuged at 280,000 g for 25

min to obtain particulate and cytosolic fractions. Saponin- and Triton X-100-soluble fractions were obtained by rocking confluent 150 mm culture dishes with 10 ml of 0.01% saponin for 5 min or 0.15% Triton X-100 for 10 min at 4°C. Following collection of the detergent-soluble fractions, the dishes were washed with PBS, the cells collected by scraping and the detergent-insoluble fractions collected by centrifugation. G proteins in the particulate and detergent-insoluble fractions were solubilized by overnight incubation in HME-PI containing 1% cholate and 1 mM dithiothreitol at 4°C. Cholate-insoluble protein was pelleted by centrifugation at 280,000 g for 25 min and the supernatant prepared for SDS-PAGE by the addition of 4 $\times$  standard SDS-PAGE sample buffer. Proteins in the cytosolic and detergent-soluble fractions were concentrated by precipitation with 12% trichloroacetic acid, washed three times with acetone and solubilized in 1 $\times$  standard SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE on a 15% polyacrylamide gel (42) and transferred to nitrocellulose using a high temperature blotting procedure (41). The nitrocellulose was immunoblotted with the anti- $\gamma_5$  antisera and visualized by autoradiography using goat anti-rabbit [ $^{125}I$ ]Ig F(ab) $_2$  fragment.

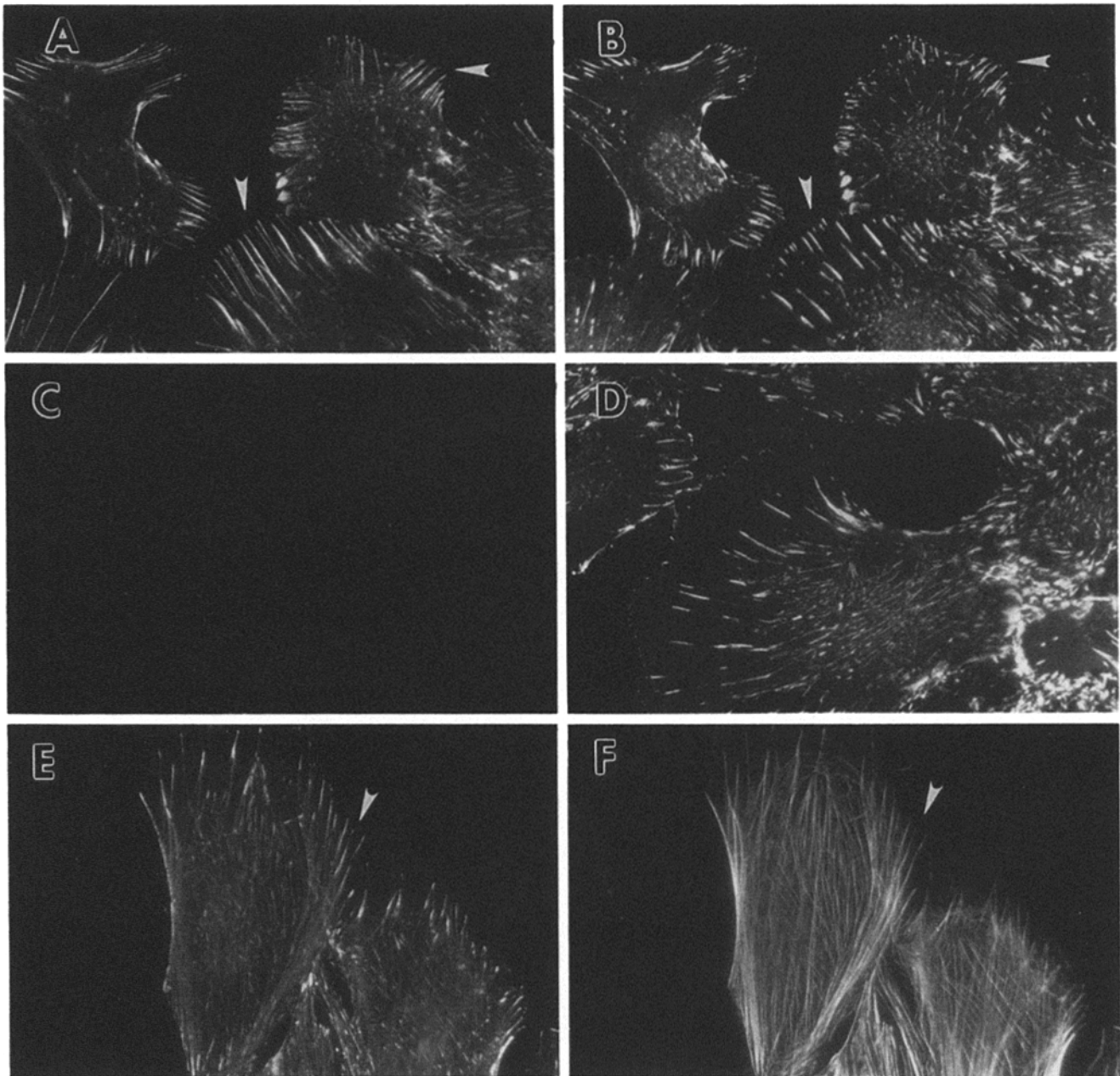
## Materials

Sprague-Dawley rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Cell culture reagents were from GIBCO-BRL (Gaithersburg, MD). Electrophoresis reagents were from Bio-Rad Laboratories (Cambridge, MA), except the nitrocellulose, which was from Micron Separations (Westborough, MA). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR). TRITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and monoclonal anti-human vinculin were from Sigma. [ $^{125}I$ ]Ig F(ab) $_2$  fragment was from New England Nuclear (Boston, MA). All other reagents were from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA).

## Results

Western immunoblots with anti-peptide antibodies specific for  $\gamma_1$ ,  $\gamma_2$ ,  $\gamma_3$ ,  $\gamma_5$ , and  $\gamma_7$  subunits have revealed the presence of the  $\gamma_3$ ,  $\gamma_5$ , and  $\gamma_7$  subunits of the heterotrimeric G proteins in cultured neonatal cardiac fibroblasts (data not shown). To determine the subcellular localization of these subunits, cells were subjected to standard immunocytochemical analysis using these same antibodies. As expected, immunostaining with anti- $\gamma_1$ - (A-4) and anti- $\gamma_2$ -specific (D-81) antisera was not observed, consistent with the inability to detect the presence of these subunits in these cells by immunoblotting. Both of these antisera have been successfully used for immunocytochemistry in cells expressing these subunits (33). The anti- $\gamma_3$ -specific antisera (B-53) produced a diffuse staining throughout the cell, which was not characterized further. The anti- $\gamma_7$ -specific antisera (A-67) did not show immunostaining above background, despite the presence of the  $\gamma_7$  subunit in fibroblasts by immunoblotting, suggesting that this antisera may not be suitable for immunocytochemical analysis. In this regard, unlike the other anti- $\gamma$  subunit antisera which were raised against  $NH_2$ - or  $COOH$  terminal sequences, the anti- $\gamma_7$  antisera was directed against a peptide antigen derived from an internal sequence and may not provide an available epitope in the native protein.

In contrast to any of the above anti- $\gamma$  subunit antisera, the anti- $\gamma_5$  antisera yielded a highly distinctive pattern of intensely fluorescent regions near the periphery of the cell that tended to protrude into the cell in a fibrous pattern (Fig. 1A). In addition, though of much less intensity, there was also a general increase in the relative fluorescence across the cell. These data indicated that a small amount of the  $\gamma_5$  subunit was distributed throughout the cell, but that most of it was highly enriched in specific regions of the cell.



**Figure 1.** Localization of the  $\gamma_5$  G protein subunit to areas of focal adhesion in neonatal cardiac fibroblasts. Cells were double stained for the  $\gamma_5$  subunit (A, C, and E) and either vinculin (B and D) or actin (E). The complete overlap of the  $\gamma_5$  subunit staining with vinculin staining indicated that  $\gamma_5$  was localized to focal adhesions. To examine specificity of immunostaining, both the anti- $\gamma_5$  and vinculin antisera were incubated overnight with 1 mg  $\gamma_5$  peptide/ml antisera at 4°C. Preincubation with  $\gamma_5$  peptide completely abolished the  $\gamma_5$  staining (C) without affecting vinculin staining (D). As shown by the arrows in A and B, the localization of  $\gamma_5$  extended beyond the focal adhesion in a fibrous pattern. E and F demonstrate that this fibrous pattern corresponded to actin fibers, as detected by phalloidin.

The pattern of intensely fluorescent regions near the periphery was suggestive of focal adhesions, which are metabolically active sites associated with cell surface-substratum interactions (28). To examine whether the anti- $\gamma_5$  subunit antisera was staining regions of focal adhesion, fibroblasts were double stained with antisera directed against the  $\gamma_5$  subunit and vinculin. Vinculin is a structural protein involved in cytoskeletal-membrane interaction in areas of focal adhesion (28). As illustrated by the arrows in Fig. 1 (A

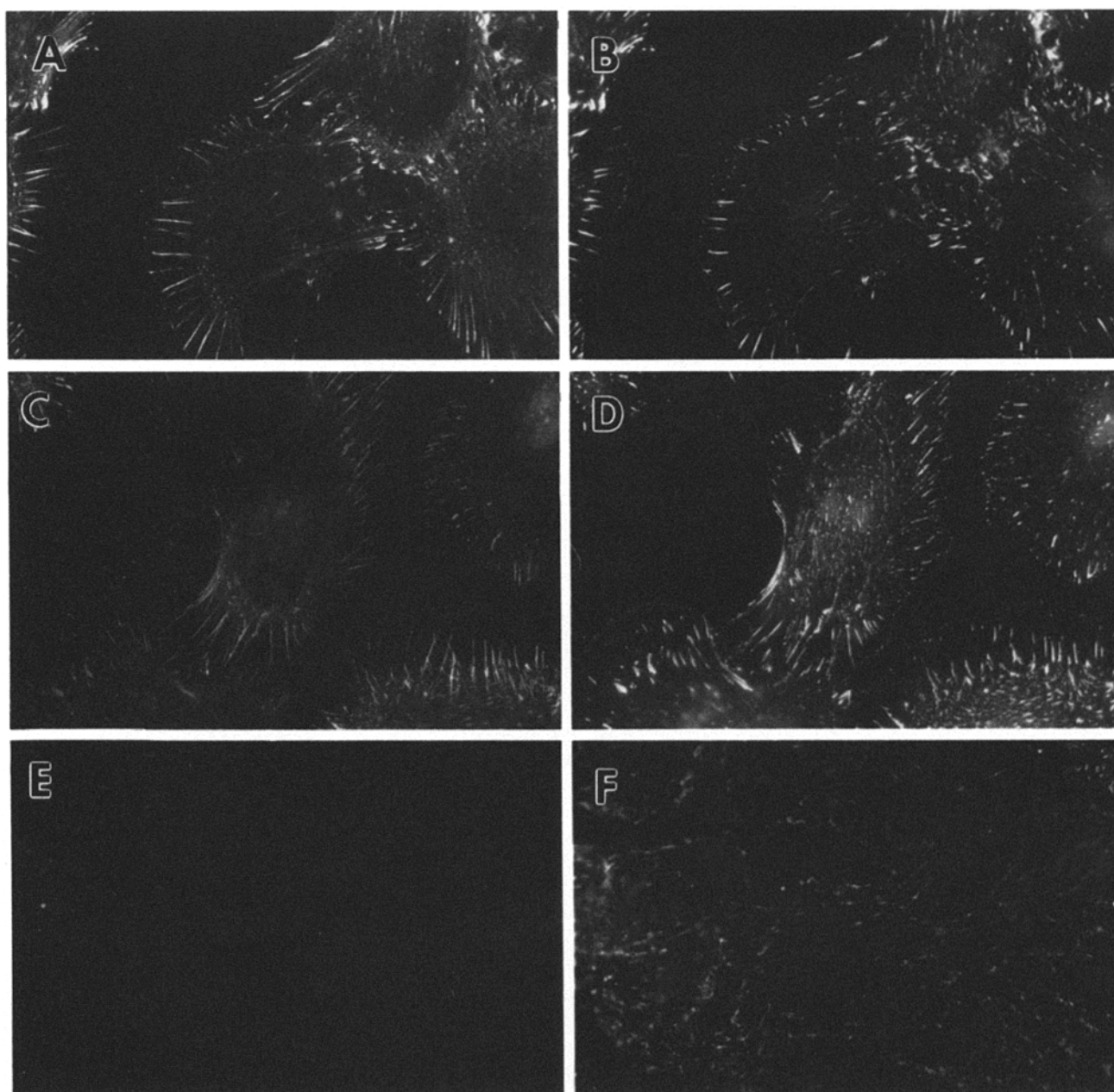
and B), the immunostaining pattern of the  $\gamma_5$  subunit showed essentially complete overlap with the staining pattern of vinculin, clearly indicating that the sites of enriched  $\gamma_5$  subunit staining corresponded to areas of focal adhesion. The specificity of the  $\gamma_5$  subunit immunostaining pattern was examined by incubating both the anti- $\gamma_5$  and the anti-vinculin antisera with the peptide used to generate the anti- $\gamma_5$  antibody. As shown in Fig. 1 (C and D), preincubation with peptide completely abolished the  $\gamma_5$  subunit immunostain-

ing, but did not affect vinculin staining. These data indicated that the  $\gamma_5$  subunit antisera specifically recognized a protein containing the peptide sequence unique to the  $\gamma_5$  subunit in regions of focal adhesion.

In addition to completely overlapping with vinculin, the  $\gamma_5$  staining often extended beyond that of vinculin, apparently stretching along the actin filaments protruding from the sites of focal adhesion. Co-localization of the  $\gamma_5$  subunit with phalloidin-stained actin showed this to be the case (Fig. 1, *E* and *F*), through the intense  $\gamma_5$  staining extended only a short distance along the actin fibers. Since sites of focal adhesion are specialized regions in which the cytoskeletal

elements couple to membrane associated signal transducing pathways and cell adhesion components, the importance of cytoskeletal integrity was investigated by disrupting actin filament association with cytochalasin B. Treatment of cells with cytochalasin B for 30 min resulted in a disintegration of vinculin, actin and the  $\gamma_5$  staining patterns. Interestingly, however, most of the  $\gamma_5$  staining remained spatially localized with the redistributed vinculin staining pattern (data not shown). Together, these data suggested that the  $\gamma_5$  subunit was specifically associated with one or more of the biochemical components that comprise focal adhesions.

While vinculin is associated with the cytoskeleton lying

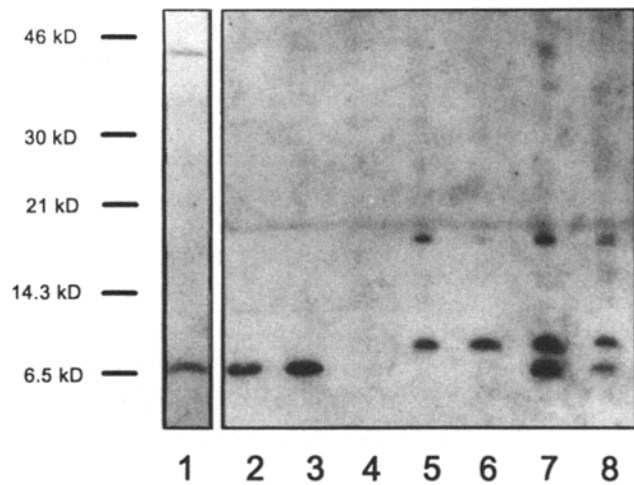


**Figure 2.** Differential detergent-extraction of the  $\gamma_5$  subunit and vinculin. Before fixation, neonatal cardiac fibroblasts were incubated on ice in PBS with no detergent (*A* and *B*), with 0.01% saponin for 5 min (*C* and *D*), or with 0.15% Triton X-100 for 10 min. After the removal of the detergent-containing media, the cells were washed with PBS and double stained for the  $\gamma_5$  subunit (*A*, *C*, and *E*) and vinculin (*B*, *D*, and *E*). Permeabilization of the cell with saponin did not affect either the  $\gamma_5$  subunit or vinculin immunofluorescence staining. Extraction of cells with Triton X-100, however, eliminated the  $\gamma_5$  staining without solubilizing vinculin.

beneath the plasma membrane, G protein  $\gamma$  subunits are generally thought to be associated with the plasma membrane. To further assess the localization of the  $\gamma_5$  subunit, cell proteins were differentially extracted with detergents. With low saponin concentrations, cells can be readily permeabilized with little solubilization of membrane-bound proteins. Thus, treatment of neonatal cardiac fibroblasts with 0.01% saponin in PBS permeabilized nearly 100% of the cells within 1 min, as assessed by loss of trypan blue exclusion. In contrast, incubation of cells with low Triton X-100 concentrations, solubilizes many membrane proteins, but poorly solubilizes cytoskeletal proteins. Compared to control cells (Fig. 2, A and B), treatment of cells with 0.01% saponin for 5 min before paraformaldehyde fixation had no effect on the co-localization of the  $\gamma_5$  subunit with vinculin (Fig. 2, C and D), indicating that the  $\gamma_5$  subunit was not freely soluble. Following exposure of cells to increasing Triton X-100 concentrations, however, the immunofluorescence detection of the  $\gamma_5$  subunit was significantly decreased with a 10-min incubation at 0.1% Triton X-100, and completely eliminated with a 10-min incubation at 0.15% Triton X-100 (Fig. 2 E). Immunofluorescent detection of vinculin, however, was not affected by a 10-min incubation at 0.15% Triton X-100 (Fig. 2 F), but required 10-min incubations with Triton X-100 concentrations of greater than 0.5% before vinculin immunofluorescence intensity was observed to decrease. Taken together, these data are consistent with a membrane localization for the  $\gamma_5$  subunit and a cytoskeletal association of vinculin.

To confirm that the loss of the  $\gamma_5$  subunit immunostaining in the Triton X-100-treated cells was due to the extraction of the  $\gamma_5$  subunit, fractions obtained from cells following differential detergent solubilization were analyzed by SDS-PAGE and immunoblotting. Neonatal cardiac fibroblasts were treated with 0.01% saponin and 0.15% Triton X-100, as described above. Proteins in the detergent-solubilized fractions were collected by precipitation in 12% trichloroacetic acid. Proteins in the insoluble fractions were solubilized by overnight incubation with 1% cholate. As shown in Fig. 3 (lane 2), the anti- $\gamma_5$  antibody detected a single major protein of 6.5 kD in the particulate fraction, which migrated with the recombinant  $\gamma_5$  standard in lane 1. No protein of similar size was detected in the cytosolic fraction (Fig. 3, lane 5). Permeabilization of cells with saponin did not solubilize the  $\gamma_5$  subunit (Fig. 3, lane 6), which remained in the saponin-insoluble fraction (Fig. 3, lane 3). In contrast, incubation of cells with 0.15% Triton X-100 completely solubilized the  $\gamma_5$  subunit, resulting in its appearance in the Triton-soluble fraction (Fig. 3, lane 7). Analysis of the Triton-insoluble fraction revealed that the  $\gamma_5$  subunit had been completely solubilized (Fig. 3, lane 4). These data confirmed that the observed changes in the immunocytochemical detection of the  $\gamma_5$  subunit with differential detergent-solubilization (Fig. 2) was due to the relative ability of the detergents to extract the  $\gamma_5$  subunit from the cells.

The immunoblotting data in Fig. 3 also provided additional confirmation of the specificity of cell staining with the anti- $\gamma_5$  antibody. In whole cell lysate (Fig. 3, lane 8), the anti- $\gamma_5$  antisera and the affinity purified anti- $\gamma_5$  antiserum (data not shown) recognized three proteins of 6.5, 9, and 18 kD. No proteins larger than 18 kD were recognized by the affinity purified anti- $\gamma_5$  antisera (data not shown). Since

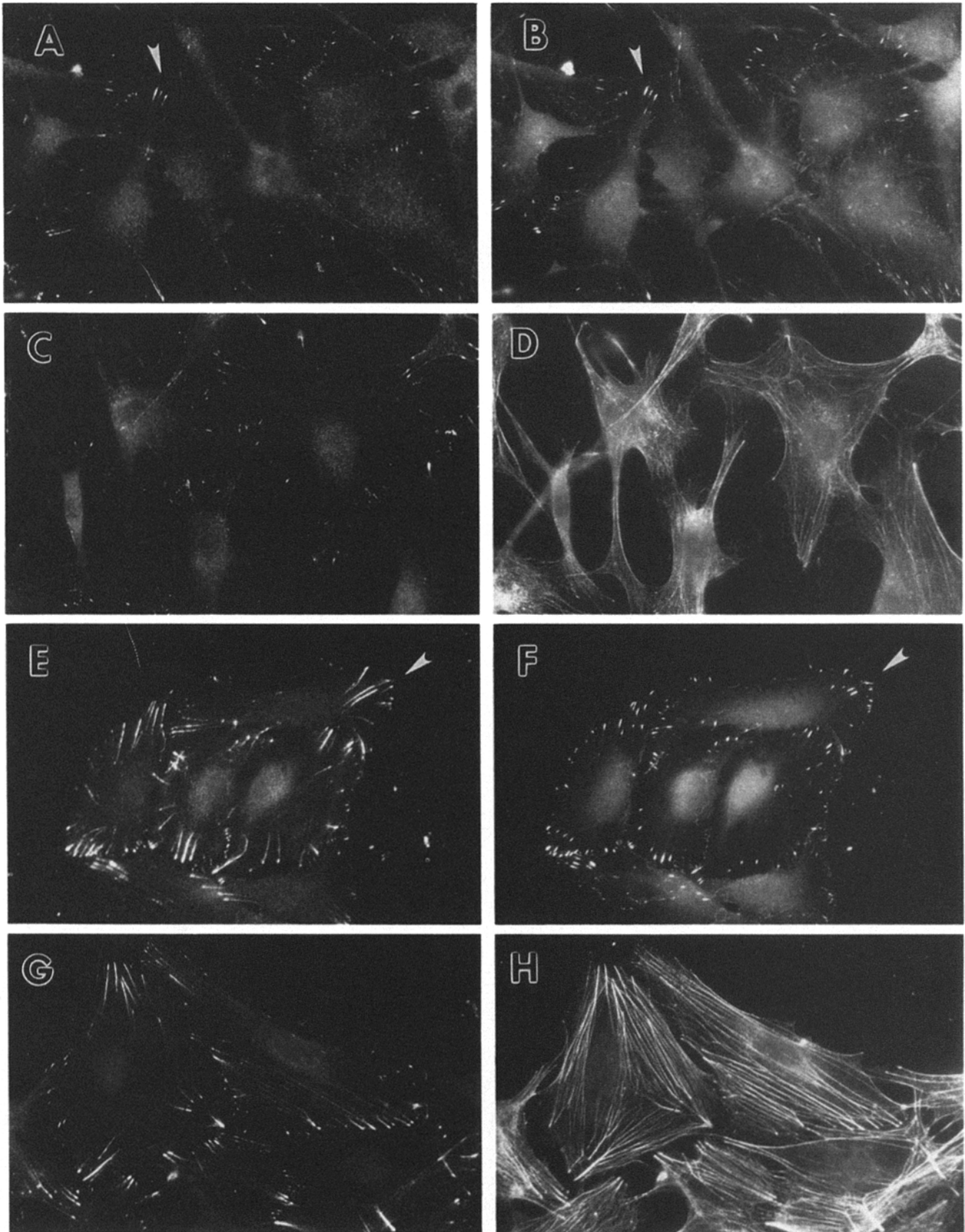


**Figure 3.** Immunoblot analysis of the  $\gamma_5$  subunit localization. Neonatal cardiac fibroblasts grown on 150 mm culture dishes were subjected to differential detergent extraction as described in Fig. 2 and processed into detergent-soluble and -insoluble fractions. The fractions were analyzed by SDS-PAGE on 15% gels and transferred to nitrocellulose as described in Materials and Methods. (Lane 1) Extract of baculovirus-infected Sf9 cells expressing  $\gamma_5$  (100  $\mu$ g); (lane 2) 1% cholate extract of control particulate fraction; (lane 3) 1% cholate extract of 0.01% saponin-insoluble protein; (lane 4) 1% cholate extract of 0.15% Triton X-100-insoluble protein; (lane 5) cytosolic fraction; (lane 6) 0.01% saponin-soluble protein; (lane 7) 0.15% Triton X-100-soluble protein; and (lane 8) whole cell lysate. Lanes 2-7 represent total proteins obtained from 50 cm<sup>2</sup> of confluent fibroblasts, while lane 8 represents 20 cm<sup>2</sup>.

recognition of the 6.5-, 9-, and 18-kD proteins by the anti- $\gamma_5$  antisera was blocked upon preincubation of the antisera with the peptide used to raise the antisera, the three proteins probably share a common epitope. However, fractionation studies indicated that only the 6.5-kD  $\gamma_5$  subunit was responsible for the immunofluorescent staining of the focal adhesions. Upon fractionation, the 9- and 18-kD proteins partitioned with the cytosolic fraction (Fig. 3, lane 5), while the 6.5-kD  $\gamma_5$  subunit remained in the particulate fraction (Fig. 3, lane 2). Consistent with this pattern of fractionation, following saponin permeabilization, the 9- and 18-kD proteins were released from the saponin-insoluble fraction, without loss of the 6.5-kD  $\gamma_5$  subunit (Fig. 3, lane 3). Since saponin permeabilization did not alter the pattern of cell staining with the anti- $\gamma_5$  antibody (Fig. 2 C), these data indicated that staining of focal adhesions with the anti- $\gamma_5$  antibody could only be attributed to recognition of the  $\gamma_5$  subunit. In addition, identical immunostaining patterns were observed with an anti- $\gamma_5$  antibody raised from a second rabbit and with the affinity-purified anti- $\gamma_5$  antibody.

To further explore the association of the  $\gamma_5$  subunit with focal adhesions, we examined a cell model system in which focal adhesions and stress fiber formation can be induced. When grown in culture, Schwann cells display a neuronal-like morphology characterized by a limited number of focal adhesion and a poorly developed actin network. However, Schwann cells transfected with the cDNA for the proteoglycan syndecan-1 have been found to exhibit increased numbers of focal adhesions and actin stress fibers (8). As shown





**Figure 4.** Recruitment of the  $\gamma_5$  subunit to focal adhesion and actin fibers in syndecan-1 expressing Schwann cells. Wild-type (A-D) and syndecan-1-expressing (E-H) Schwann cells were double stained for the  $\gamma_5$  subunit (A, C, E, and G) and either vinculin (B and F) or actin (D and H). In wild-type cells, the pattern of  $\gamma_5$  subunit staining (A) was essentially identical to vinculin staining (B), with little extension along a poorly developed actin fiber network (C and D). In syndecan-1-expressing cells, there was an increase in the number and intensity of vinculin-staining sites (F) and the actin fiber network was well developed (H). This was associated with an enriched  $\gamma_5$  subunit staining pattern that not only localized with vinculin (E), but also extended along the actin filaments (G), as observed in the fibroblasts.

in Fig. 4, (A and B), double stained wild-type Schwann cells showed diffuse patterns of  $\gamma_5$  subunit and vinculin staining, with a small number of regions with enhanced and overlapping staining (see *arrows*). Unlike the fibroblasts (Fig. 1, A and B), the regions of enriched  $\gamma_5$  staining did not extend beyond that of vinculin staining, perhaps due to the poorly developed actin network, as shown by phalloidin staining (Fig. 4 D). These data suggested that the localization of the  $\gamma_5$  subunit to areas of focal adhesion was not necessarily dependent on the presence of actin stress fibers. In syndecan-1-transfected Schwann cells, the number and intensity of regions of overlapping  $\gamma_5$  subunit and vinculin staining were greatly increased (Fig. 4, E and F), relative to wild-type cells. In this case, like the fibroblasts, the  $\gamma_5$  staining also extended out from the focal adhesion sites along a well-developed actin network (Fig. 4, G and H). In fact,  $\gamma_5$  staining can be observed as a punctate pattern along entire filaments. These data clearly suggested that the  $\gamma_5$  subunit apparently associates with components of focal adhesions and with components distributed along actin stress fibers.

Since the localization of the  $\gamma_5$  subunit to focal adhesion regions has significant ramifications for G protein-coupled signaling pathways, it was important to establish whether this association was broadly observed. As shown in Fig. 5 (A and B), the  $\gamma_5$  subunit co-localized with vinculin in cultured rat aortic smooth muscle cells, which form focal adhesions *in vivo* and in COS cells (data not shown). In addition, the time course of focal adhesion formation in neonatal cardiac fibroblast, freshly plated onto fibronectin-coated coverslips, indicated that the  $\gamma_5$  subunit localized to sites of focal adhesions as rapidly as vinculin (data not shown). Taken together, these data underscore the generality and physiological relevance of a  $\gamma_5$  subunit association with focal adhesions.

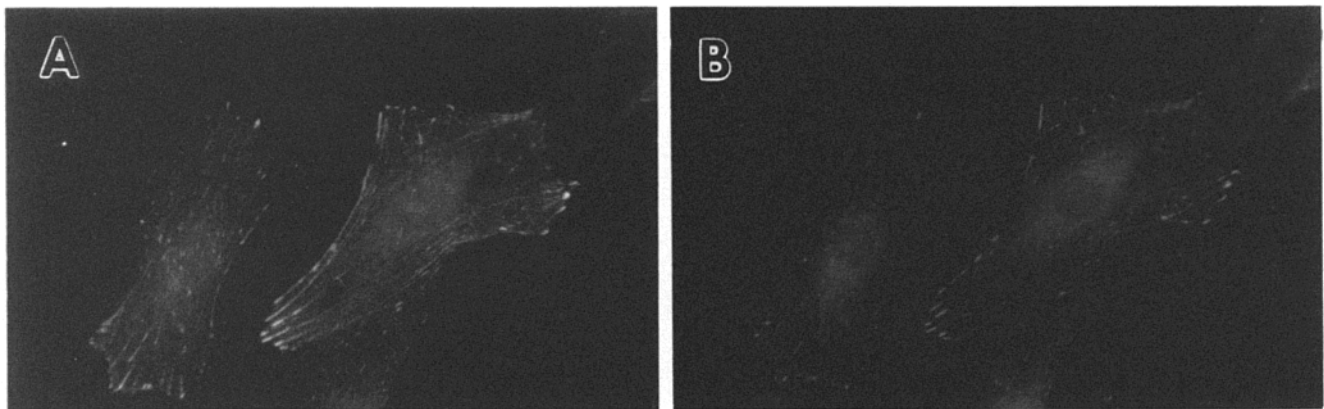
## Discussion

Signal transducing heterotrimeric G proteins are responsible for coupling a large number of cell surface receptors to the appropriate effector(s). Examining the location of the  $\gamma_5$  subunit in neonatal cardiac fibroblasts with a  $\gamma_5$  subunit-specific anti-peptide antibody, yielded a highly distinctive

pattern of intensely fluorescent regions near the periphery of the cell that tended to protrude into the cell in a fibrous pattern. Double staining with an anti-vinculin antibody and phalloidin indicated that the  $\gamma_5$  subunit co-localized with the focal adhesion protein vinculin and that the  $\gamma_5$  subunit immunofluorescence extending out from the vinculin pattern was along actin fibers. These data clearly indicated that the  $\gamma_5$  subunit of the heterotrimeric G proteins was highly enriched in regions of focal adhesion and along associated stress fibers. Similar data were obtained in rat aortic smooth muscle cells and Schwann cells, demonstrating that the association of the  $\gamma_5$  subunit with areas of focal adhesion was widespread. While it has been known that regions of focal adhesion are important signal transduction sites, these data suggest that a significant complement of heterotrimeric G protein-coupled signaling pathways involving the  $\gamma_5$  subunit are concentrated at sites of focal adhesion.

Recent evidence supports a growing importance of the  $\gamma$  subunit in heterotrimeric G protein structure and function. Comparison of the  $\gamma$  subunits has revealed a large degree of structural diversity, with the five known  $\gamma$  subunits sharing only 25-70% identities (5). Thus, the  $\gamma$  subunits display a degree of structural heterogeneity similar to that found between  $\alpha$  subunits, and, since the  $\gamma$  subunits directly interact with the  $\alpha$  subunits (38),  $\alpha$ - $\gamma$  interactions may impose structural specifications for assembly of particular  $\alpha\beta\gamma$  complexes. With regard to G protein function, selective "knock-out" of specific  $\gamma$  subunits has been shown to differentially eliminate somatostatin and muscarinic receptors coupling to inhibition of  $\text{Ca}^{2+}$  channels (24, 25). Another feature of heterotrimeric G protein  $\gamma$  subunits is that they contained COOH-terminal CAAX boxes and are accordingly, appropriately isoprenylated (29, 31, 49). While the functional consequences of protein prenylation are complex, prenylation appears to be involved in membrane localization, perhaps via a membrane-bound "docking protein" (18, 21, 30, 32). Particularly since the COOH-terminal region of the  $\gamma_5$  subunit is a region of high structural diversity, a membrane, and perhaps focal adhesion targeting function for the  $\gamma_5$  subunit is a reasonable hypothesis.

At present, we can only speculate as to the functional role of the  $\gamma_5$  subunit in focal adhesions. Co-localization of spe-



**Figure 5.** Co-localization of the  $\gamma_5$  subunit and vinculin in rat aortic smooth muscle cells. Rat aortic smooth muscle cells have been shown to exhibit focal contacts *in vivo*. The co-localization of the  $\gamma_5$  subunit with vinculin in these cells indicate that the association of the  $\gamma_5$  subunit with focal adhesions and actin stress fibers is widespread and likely to occur *in vivo*.

cific  $\alpha$ ,  $\beta$ , and  $\gamma_5$  subunits may reflect preferential assembly of heterotrimeric G proteins uniquely suited to particular signaling pathways in focal adhesions. A preliminary screen with antibodies specific for  $\alpha_6$ ,  $\alpha_{i1-3}$ , and  $\alpha_{q11}$  resulted in cell staining above background, but the pattern of staining was generally diffuse. In the case of  $\alpha_6$ , a weak staining was observed along actin stress fibers. There are several possible interpretations for these preliminary data. The  $\alpha$  subunit associated with the  $\gamma_5$  subunit was one other than those screened, perhaps an undescribed novel  $\alpha$  subunit, since evidence for additional isoforms for all of the heterotrimeric G protein subunits exists. On the other hand, the preliminary data obtained with the above anti- $\alpha$  antisera may be misleading, due to any of the potential problems related to using anti-peptide antibodies that have not been fully characterized for use in immunocytochemistry. Alternatively, a third, and most intriguing possibility, is that the  $\gamma_5$  subunit is a "targeting" subunit for focal adhesion-associated heterotrimeric G proteins, perhaps recruiting many different  $\alpha$  subunits to form a variety of  $\alpha_x\beta_x\gamma_5$  heterotrimers.

Traditionally, focal adhesions are sites where transmembrane integrin molecules bind extracellular matrix proteins, such as fibronectin and vitronectin, to cytoskeletal proteins that ultimately are associated with actin stress fibers (28). The most studied focal adhesion integrin, the fibronectin receptor, directly interacts with the cytoskeletal proteins talin and  $\alpha$ -actinin, which, together with several other proteins, form an actin fiber nucleation site. While the extension of the  $\gamma_5$  subunit staining along the actin fiber was initially thought to be reminiscent of  $\alpha$ -actinin localization, dual staining of the cardiac fibroblasts revealed that the  $\alpha$ -actinin staining was uniform along the entire distance of the actin fiber (data not shown), while the  $\gamma_5$  subunit staining diminished along the same fiber as it protruded from the focal adhesion. In this regard, the  $\gamma_5$  subunit staining pattern appeared to be similar to that of zyxin (10), a cytoskeletal protein that directly interacts with  $\alpha$ -actinin. In any case, since  $\alpha$ -actinin, talin, vinculin, and zyxin are thought to be involved in cytoskeletal-membrane interactions, the co-localization of the  $\gamma_5$  subunit with these proteins is highly suggestive of G protein-mediated regulatory inputs.

While it has been known for some time that cell movement is, in general, inversely related to the extent of focal adhesion formation, recent data have suggested that focal adhesions are metabolically active areas involved in regulation of both cell adhesion and cell proliferation (4, 44, 50). The focal adhesion tyrosine kinase, pp125<sup>FAK</sup>, protein kinase C- $\alpha$ , pp60<sup>src</sup> and other protein kinases implemented in the control of cell growth have been localized to focal adhesions. In Swiss 3T3 cells, stimulation with bombesin, vasopressin or endothelin resulted in the rapid tyrosine phosphorylation of pp125<sup>FAK</sup> and other focal adhesion associated proteins (50). The fact that the receptors for these hormones are heterotrimeric G protein-coupled receptors and the demonstration that the  $\gamma_5$  subunit of heterotrimeric G proteins is enriched in focal adhesions suggests that these receptors may also be localized in regions of focal adhesions. Furthermore, G proteins may also be involved in mediating integrin receptor signaling. Arcangeli et al. (1) recently demonstrated that pertussis toxin abolished adhesion induced hyperpolarization and neurite outgrowth in neuroblastoma cells, suggesting that a G protein was interposed between integrin receptors and activation

of a cell adhesion-activated K<sup>+</sup> channel. The localization of the  $\gamma_5$  subunit to regions of focal adhesion clearly suggests that this subunit may play a central role in G protein coupling signaling pathways present in focal adhesions.

Clearly, data are emerging in support of the postulate that focal adhesions are sites of convergence of signaling pathways regulating cell adhesion and cell growth (20, 50). The localization of the  $\gamma_5$  subunit to sites of focal adhesion indicates that G protein-coupled signaling pathways spatially overlap with cell adhesion-mediated signaling pathways. Hence, cell signaling pathways regulating pp125<sup>FAK</sup>, protein kinase C- $\alpha$ , pp60<sup>src</sup>, and other protein kinases are likely to be intermixed with G protein coupled effectors, which include, adenylyl cyclases, phospholipase A<sub>2</sub>, the  $\beta$  family of phospholipase C, receptor kinases, as well as Ca<sup>2+</sup> and K<sup>+</sup> channels. Future studies must be aimed at addressing how extracellular matrix signals interact with more traditional hormone-mediated signaling pathways to coordinately regulate cell function. Whether the receptor systems represent independent pathways that activate common effectors or whether they act as a coreceptor (20) system, which must synergize to induce the appropriate biological response, remains to be established.

We thank Tom Smink, Eric Balcueva, and Rich Stahl for their skillful technical assistance and Dr. Paul Sternweis for generously supplying the  $\alpha_6$  and  $\alpha_{q11}$  antisera.

This work was supported by National Institutes of Health grants DK 45417 to C. A. Hansen, HL 49278 and GM 39867 to J. D. Robishaw, and NS 21925 to D. J. Carey.

Received for publication 25 January 1994 and in revised form 27 April 1994.

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