CELL REGULATION, Vol. 2, 1097–1113, December 1991

Subcellular distribution of the α subunit(s) of G_i: visualization by immunofluorescent and immunogold labeling

Jean M. Lewis,*† Marilyn J. Woolkalis,† George L. Gerton,‡ Robert M. Smith,§ Leonard Jarett,§ and David R. Manning†
*Graduate Group in Biochemistry
Departments of †Pharmacology,
‡Obstetrics and Gynecology
§Pathology and Laboratory Medicine
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

The subcellular distribution of the α subunit(s) of G_i has an obvious bearing on the ability of this protein to interact with receptors and targets and on its potential to serve in still unexplored capacities. In this study, we have examined the distribution of $G_{i\alpha}$ by means of light and electron microscopy. The cells employed were mouse 3T3 fibroblasts, normal rat kidney fibroblasts, rat C6 glioma cells, human umbilical vein endothelial cells, and human 293 kidney fibroblasts. By indirect immunofluorescence, two patterns of $G_{i\alpha}$ were evident. The more prominent was that associated with phase-dense, cytoplasmic structures exhibiting a tubule-like morphology. A similar distribution was noted for mitochondria, indicating attachment to a subset of microtubules. The second pattern appeared as a diffuse, particulate fluorescence associated with the plasma membrane. By immunogold labeling and electron microscopy, two populations of $G_{i\alpha}$ were again evident. In this instance, labeling of the plasma membrane was the more prominent. Gold particles were most often evenly distributed along the plasma membrane and were concentrated along microspikes. The second, less abundant population of $G_{i\alpha}$ represented the subunit (or fragments) within lysosomes. Specificity in immunolabeling was confirmed in all instances by immunotransfer blotting, the use of antibodies differing in specificities for epitopes within $G_{i\alpha}$, the absence of labeling with preimmune sera, and the decrease in labeling after preincubation of antisera with appropriate peptides. These results support the proposal that several populations of $G_{i\alpha}$ exist: those evident within the cytoplasm by immunofluorescence, those present at the plasma membrane, and those evident within lysosomes by immunogold labeling.

Introduction

The ability of cells to proliferate and perform specialized tasks is dependent on the precise orchestration of external and internal signals. GTP-binding regulatory proteins (G proteins)[1] participate in many of these events by conducting the flow of information from receptors to target enzymes or channels (Freissmuth *et al.,* 1989; Birnbaumer, 1990). G proteins are $\alpha\beta\gamma$ heterotrimers, whose identities are most often equated with those of the α subunits (G_α). Currently recognized G_α include one to four subtypes each of $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, $G_{t\alpha}$, $G_{z\alpha}$, $G_{q\alpha}$, and $G_{12\alpha}$. These subunits individually regulate different effectors, including adenylyl cyclase, phospholipase C, cGMP-selective phosphodiesterase, and certain types of Na^+, K^+, and Ca^{2+} channels.

G proteins are often portrayed on the inner aspect of the plasma membrane, reflecting the view that elements relevant to signal transduction operate in apposition to the cell surface. Information obtained through purification of membranes is indeed consistent with the plasma membrane as a site of G proteins (Scherer *et al.,* 1987; Bokoch *et al.,* 1988). The existence of G proteins elsewhere, however, has become the subject of considerable interest. The different G_α are hydrophilic (Huff *et al.,* 1985; Sternweis, 1986), a property suggesting the potential for translocation from membranes to the cytosol. Several cytoplasmic phenomena are sensitive to nonhydrolyzable analogues of GTP, including targeting of proteins to the endoplasmic reticulum (Connolly and Gilmore, 1989), degranulation (Gomperts and Tatham, 1988), and recognition or processing of vesicles

[1] Abbreviations used: BSA, bovine serum albumin; G protein, GTP-binding regulatory protein; KLH, Keyhole limpet hemocyanin; LAMP, lysosome-associated membrane protein; PBS, phosphate-buffered saline; PNS, postnuclear supernatant.

(Melançon *et al.*, 1987; Balch, 1989). Toxin-catalyzed radiolabeling has revealed that populations of G proteins are associated with various subcellular structures that can be resolved by density-gradient centrifugation. In neutrophils, for example, $G_{i\alpha}$ has been detected by pertussis toxin-catalyzed ADP-ribosylation not only in the plasma membrane but in the cytosol (Bokoch *et al.*, 1988) and in specific granules (Rotrosen *et al.*, 1988). $G_{i\alpha}$ identified by similar means is the only one of several G_α to cosediment with the sarcoplasmic reticulum of canine heart (Scherer *et al.*, 1987) and has been localized to the rough endoplasmic reticulum of canine pancreas (Audigier *et al.*, 1988).

Significant evidence exists for constraints in the access of receptors to G proteins within the plasma membrane. Heterogeneity in the affinities of particular receptors for agonists suggests that large populations of receptors are normally uncoupled from G proteins, despite relative excesses of the latter (DeLean *et al.*, 1980; Neubig *et al.*, 1985). Differences among agonists in the ability to attenuate toxin-catalyzed ADP-ribosylation of G_α also argue the potential for segregation of receptors and G proteins (Brass *et al.*, 1988). Receptors and G proteins are both subject to post-translational modifications that may alter communication, such as phosphorylation (Sibley *et al.*, 1987; Carlson *et al.*, 1989). Receptors can also undergo translocation into regions of membrane apparently deficient in G proteins through internalization (Waldo *et al.*, 1983) or lateral sequestration (Jesaitis *et al.*, 1989).

Despite interest in the spatial organization of G proteins, immunocytochemical data are limited. Indirect immunofluorescence has been used to establish the existence of $G_{t\alpha1}$ and $G_{t\alpha2}$ within retinal rod outer segments and cones, respectively (Lerea *et al.*, 1986). Similar methods have revealed the presence of $G_{t\alpha}$-, $G_{o\alpha}$-, and/or $G_{i\alpha}$-like proteins associated with sperm acrosomes (Garty *et al.*, 1988; Glassner *et al.*, 1991). Particularly high levels of $G_{o\alpha}$ are evident at the tips of processes extending from rat PC12 cells (Strittmatter *et al.*, 1990) and along the apical aspect (i.e., microvilli) of rat ependymal cells (Péraldi *et al.*, 1989). $G_{i\alpha2}$ within polarized kidney epithelial cells can exist at the basolateral membrane, whereas $G_{i\alpha3}$ may codistribute with the Golgi apparatus (Ercolani *et al.*, 1990). $G_{z\alpha}$ within rat brain is constrained to neurons and by electron microscopy appears to be localized predominantly to cytoplasmic structures (e.g., nuclear membranes and endoplasmic reticulum) (Hinton *et al.*, 1990).

Spatial distributions of G proteins can serve as determinants in the response of a cell to agonists and may additionally reflect still unexplored functions. In this study, therefore, we have investigated the subcellular distribution of the G protein G_i, and specifically the one or more forms of $G_{i\alpha}$ in mouse 3T3 fibroblasts and other types of cultured cells. Our analysis entailed the use of both immunofluorescence and immunogold labeling in conjunction with light and electron microscopy. We report here the existence of several populations of $G_{i\alpha}$. The most prominent population by immunofluorescence is cytoplasmic and probably represents structures existing along subsets of microtubules. Immunogold labeling reveals low levels of $G_{i\alpha}$ within lysosomes and particularly high concentrations along microspikes of the plasma membrane.

Results

Development of antibodies

Most antibodies used in this study were generated in rabbits against Keyhole limpet hemocyanin (KLH)-conjugated peptides (Table 1). By immunotransfer blotting, antibodies within antiserum 1398 recognize $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ equivalently (Carlson *et al.*, 1989). A similar pattern of recognition is evident for 8645 and 1013. Antibodies within 8729 exhibit a preference for $G_{i\alpha1}$ and $G_{i\alpha2}$, whereas those of 3646, 1521, and 1518 are selective for $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$, respectively (Williams *et al.*, 1990). The term $G_{i\alpha}$ will be used here to refer to the one or more subtypes of $G_{i\alpha}$ without distinction.

The potential of antibodies to react with cellular proteins was first assessed by immunotransfer blotting. The results shown in Figure 1 were obtained with antiserum 8729 and preimmune serum, using as sources of protein partially purified membranes (50K Pellet, left and middle panels) and fractions corresponding to a low-speed pellet (1K P), partially purified membranes (50K P), and cytosol (50K S) (right panel). By this type of analysis, antibodies within 8729, but not preimmune serum, clearly detect a 40- to 41-kDa protein(s). This protein is $G_{i\alpha}$ and is evident in both partially purified membranes and the low-speed pellet. In partially purified membranes it represents ~0.2% of protein. Recognition of the subunit by 8729 is highly selective, as it is for the other $G_{i\alpha}$-directed antisera used in this study.

Light microscopy

Immunofluorescence microscopy was undertaken with antiserum 8729 and mouse Swiss

Table 1. Antibodies of specificity for $G_{i\alpha}$

Antiserum	Antigen[a]	Recognition
1398	*C*GAGESGKSTIVKQMK	$G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$
8645	*C*FDVGGQRSERKK	$G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$[b]
1013	*C*FEDLNKRKDTKE	$G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$
8729[c]	KNNLKDCGLF	$G_{i\alpha1}$, $G_{i\alpha2}$ ($G_{i\alpha3}$[d])
3646	*C*LDRIAQPNYI	$G_{i\alpha1}$
1521	*C*DLERIAQSDYI	$G_{i\alpha2}$[b]
1518	*C*IDFGDAARADDAR	$G_{i\alpha3}$
5296	G_i/G_O	$G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{O\alpha}$, G_β

Antibodies were generated in rabbits by injection of KLH-conjugated peptides or a combination of G_i and G_o. Recognition of the different subtypes was assessed by immunotransfer blotting with subunits encoded by rat cDNA and expressed in *E. coli* (Carlson *et al.*, 1989; Williams *et al.*, 1990). Sequences for $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ are provided by Jones and Reed (1987). Parentheses refer to less than equivalent, but detectable, cross-reactivities.

[a] *C* refers to an *N*-terminal cysteine attached to facilitate coupling via *m*-maleimidobenzoyl-N-hydroxysuccinimide ester.

[b] Antisera 8645 and 1521 also recognize $G_{z\alpha}$ (Carlson *et al.*, 1989) and 8645 additionally recognizes $G_{q\alpha}$ or $G_{11\alpha}$ (Taylor et al., 1991). $G_{z\alpha}$ is not detected by immunotransfer blotting in the cells used in this study.

[c] Antiserum 8729 was generated with the same peptide as previously described in the generation of antiserum 8730 (Carlson *et al.*, 1989). The two antisera provide equivalent results.

[d] The corresponding sequence in $G_{i\alpha3}$ is KNNLKECGLY.

3T3 fibroblasts. The 3T3 fibroblasts contain $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ in an ~2:10:1 ratio, as determined by immunotransfer blotting with subtype-selective antibodies and isoelectric focusing of immunoprecipitates prepared with 1398 (not shown). When fibroblasts were fixed, permeabilized, and incubated sequentially with 8729 and fluorescein-conjugated goat anti-rabbit IgG, two components of fluorescence were evident (Figure 2B). The first, and more prominent, corresponded to tubule-like structures present within the cytoplasm. These structures were particularly abundant in the perinuclear region, and on occasion each could be resolved into smaller particles (<0.4-μm diameter) held in tandem. The second component of fluorescence was a faint, particulate pattern at the plasma membrane. Both components were observed with IgG purified from 8729 by affinity chromatography on either immobilized protein A or immobilized peptide. Neither component was detected with preimmune serum (Figure 2C), with 8729 preincubated with either the peptide against which it was generated (Figure 2D) or recombinant $G_{i\alpha}$ or with antibodies to irrelevant peptides. Preincubation of 8729 with irrelevant peptides, recombinant $G_{o\alpha}$, or KLH alone had no effect on what was normally seen with this antiserum. Patterns of fluorescence observed for 8729 were identical whether fluorescein- or rhodamine-conjugated secondary antibodies were used. Equivalent patterns were also observed by phase-contrast microscopy using horseradish peroxidase-based methods of immunolabeling.

The results obtained were relatively independent of methods of fixation and permeabilization. Protocols most often included treatment of cells with 4% paraformaldehyde followed by 0.2% Triton X-100. The same patterns of fluorescence were obtained after fixation with 95% methanol/5% acetic acid at room temperature or acetone at −20°C or substitution of 0.2% saponin for Triton X-100. Detergents were always required when paraformaldehyde was used, most likely reflecting the need to permeabilize cells for the antibodies to gain access to the antigen. Immunolabeling by 8729 (and other $G_{i\alpha}$-directed antibodies) could not be achieved using 0.5% glutaraldehyde, regardless of detergent, perhaps due to inaccessibility through extensive cross-linking of plasma membrane protein. The association of immunoreactive protein with structures in the cytoplasm was confirmed by confocal microscopy.

Patterns similar to those obtained for 3T3 fibroblasts were observed with normal rat kidney fibroblasts, rat C6 glioma cells, and human umbilical vein endothelial cells (Figure 3). For each of these, $G_{i\alpha2}$ is the most abundant form of $G_{i\alpha}$

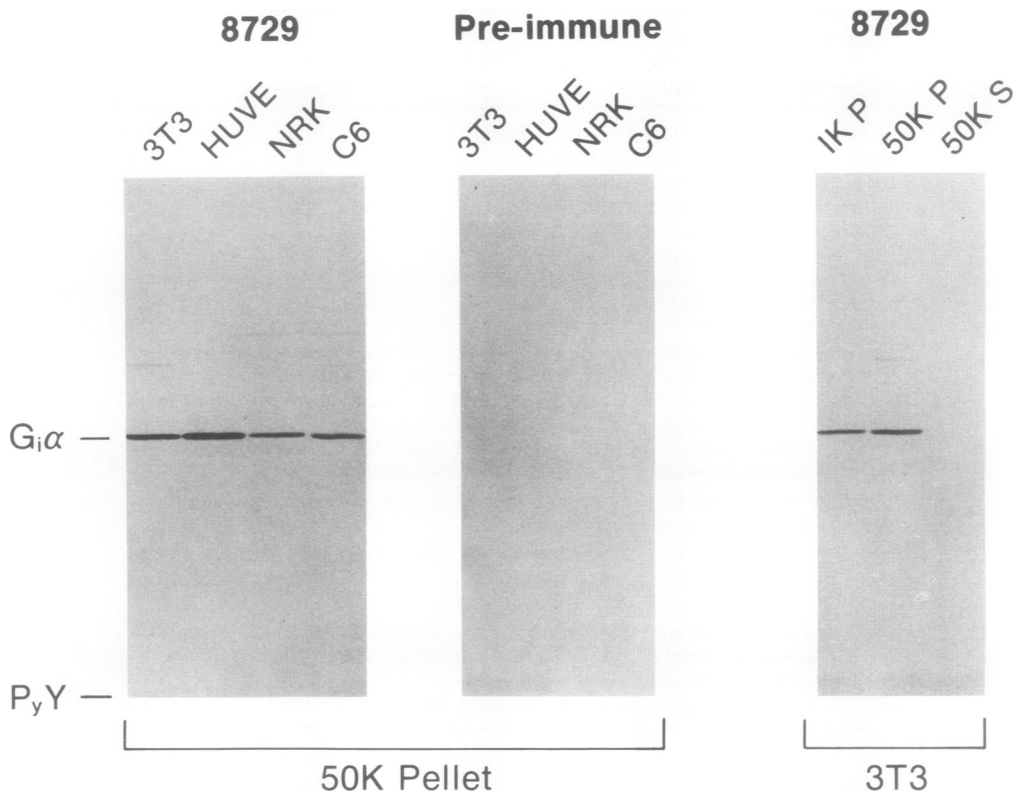


***Figure 1.* Analysis of cell fractions for $G_{i\alpha}$ by immunotransfer blotting.** Cells indicated were lysed by homogenization in hypotonic medium, then subjected to sequential low- and high-speed centrifugation to yield high-speed pellets (50K Pellet; left and middle). Low- (1K P) and high- (50K P) speed pellets and supernatant (50K S) are shown for 3T3 fibroblasts (right). Proteins within the indicated fractions (50 μg/lane) were resolved by SDS-PAGE, then transferred to nitrocellulose for immunostaining with antiserum 8729 or preimmune serum. The mobility of $G_{i\alpha}$ purified from rabbit liver is indicated; PyY refers to the pyronin Y used to monitor transfer. Cells are: 3T3, mouse 3T3 fibroblasts; HUVE, human umbilical vein endothelial cells; NRK, normal rat kidney fibroblasts; and C6, rat C6 glioma cells.

with 5- to 10-fold less $G_{i\alpha 3}$ (kidney and endothelial cells) or $G_{i\alpha 1}$ (glioma cells) (data not shown). Again, the predominant component of fluorescence for each cell was associated with structures distinct from the plasma membrane. Slight variations in the intricacy of the patterns among cells were noted.

Patterns of fluorescence nearly identical to those observed with antiserum 8729 were obtained using 8645, 1013, 1521, and 1518 (e.g., Figure 4, C and E for 8645 and 1521) with variations occurring in intensity and degree of nonspecific background. Thus, antibodies specific for different epitopes within $G_{i\alpha}$ corroborate the largely cytoplasmic distribution observed with 8729. An antiserum generated in rabbits against a combination of bovine brain G_o and G_i (i.e., 5296) also produced the same patterns (Figure 4B) as did an antiserum generated in a mouse against rabbit liver G_i (not shown). For the $G_{i\alpha 1}$-specific antiserum 3646, the pattern of fluorescence viewed for 3T3 cells was somewhat different from that observed with the other antisera (Figure 4D). The immunofluorescence in this case was distributed in a punctate and often asymmetric fashion about the nucleus, with labeling detected in only ~20% of the cells.

Human kidney 293 cells were found by immunotransfer blotting to contain about one-tenth the amount of $G_{i\alpha 2}$ present in the other cells (data not shown). Not unexpectedly, the intensity of immunofluorescence obtained for these cells with the $G_{i\alpha 2}$-selective antiserum 1521 was far less than that observed in other cells (Figure 4, compare E and F). The low level of nuclear fluorescence observed in 293 cells with 1521 was also evident with the preimmune serum.

Among antisera that did not support immunofluorescence was 1398, which is specific for all three forms of $G_{i\alpha}$. The inability of 1398 to provide a signal was initially surprising because this antiserum is one of the most effective in protocols of immunotransfer blotting and im-

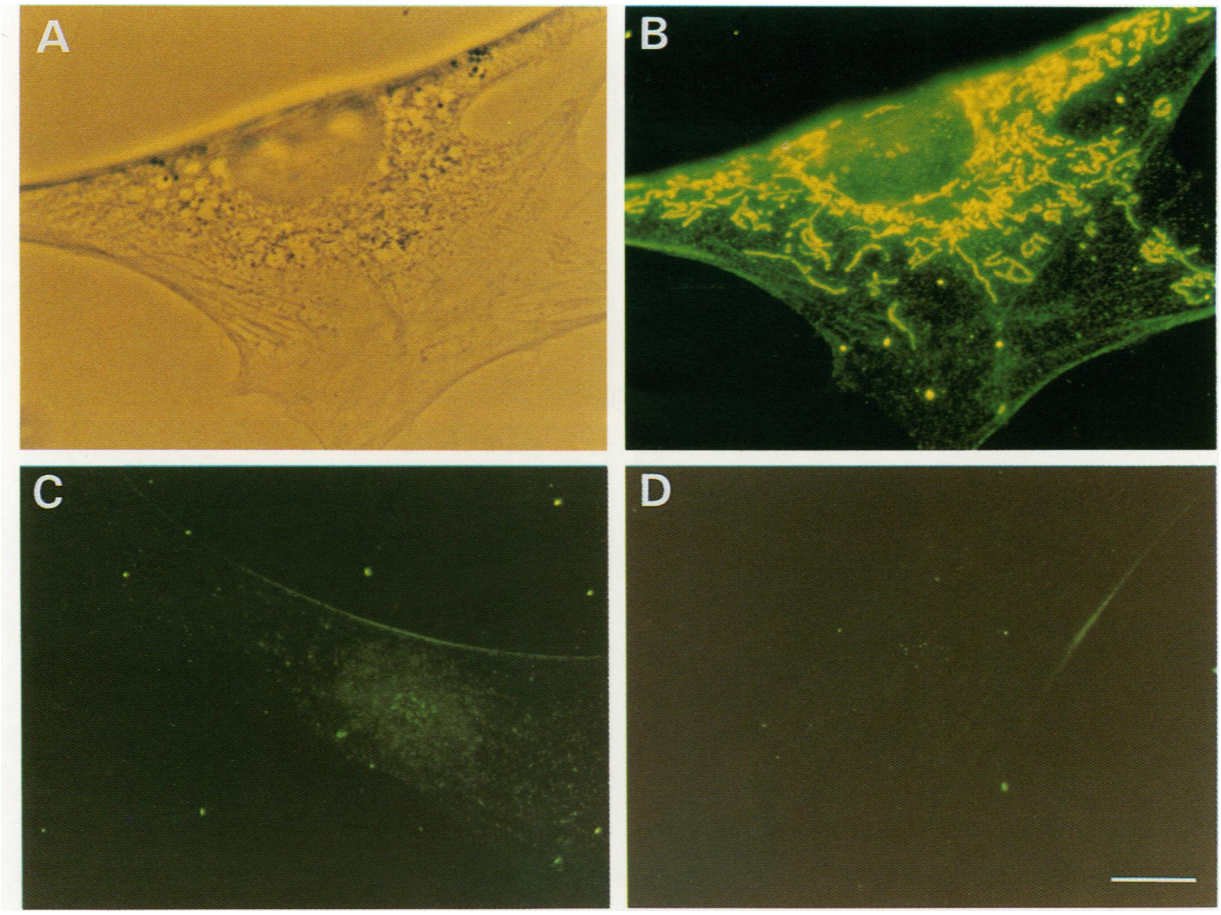


Figure 2. **Indirect immunofluorescence for mouse 3T3 fibroblasts using antiserum 8729.** (A and B) Paraformaldehyde-fixed, permeabilized mouse 3T3 fibroblasts were incubated sequentially with antiserum 8729 (1:100) and fluorescein-conjugated goat anti-rabbit IgG (1:250), then viewed by (A) phase-contrast or (B) fluorescence microscopy. (C and D) Fluorescence microscopy utilizing (C) preimmune serum or (D) antiserum 8729 preincubated with 10 μg/ml of the peptide KNNLKDCGLF at 4°C for 16 h. Bar, 10 μm.

munoprecipitation (Carlson *et al.,* 1989; Williams *et al.,* 1990). Preliminary experiments were undertaken with several G_{β}-directed antibodies (Law *et al.,* 1991), but consistent patterns of immunofluorescence were not obtained.

Efforts were made to identify more precisely the cytoplasmic structures with which the $G_{i\alpha}$-related fluorescence was associated. An obvious correspondence to structures identified with antibodies specific for actin, clathrin-coated vesicles, and the Golgi apparatus (e.g., Figure 5B for the Golgi apparatus) was not observed. However, a similarity in the immunofluorescence to patterns established by mitochondria (Figure 5C) was notable. The mitochondria here, as in other cells, are aligned along microtubules (Goldstein and Korczack, 1981). Double labeling confirmed the colocalization of $G_{i\alpha}$ with mitochondria in human endothelial cells (Figure 5, E and F). The pattern observed for $G_{i\alpha}$ also resembled distributions reported for lysosomes in certain types of cells or under unusual conditions of fixation (i.e., "tubular lysosomes") (Phaire-Washington *et al.,* 1980; Swanson *et al.,* 1987; Heuser, 1989). A lysosomal identity, however, was not convincingly supported by immunofluorescence labeling of lysosome-associated membrane proteins lysosome-associated membrane protein (LAMPs)-1 or -2 (Figure 5D) or by vital staining with lucifer yellow (not shown).

Attempts to demonstrate an association of $G_{i\alpha}$ with mitochondria per se were unsuccessful. Fractions of 3T3 fibroblasts enriched in mitochondria (17/35% Metrizamide interface [Storrie and Madden, 1990]) were found to contain only small amounts of the subunit when assayed by immunotransfer blotting (<10% total). The

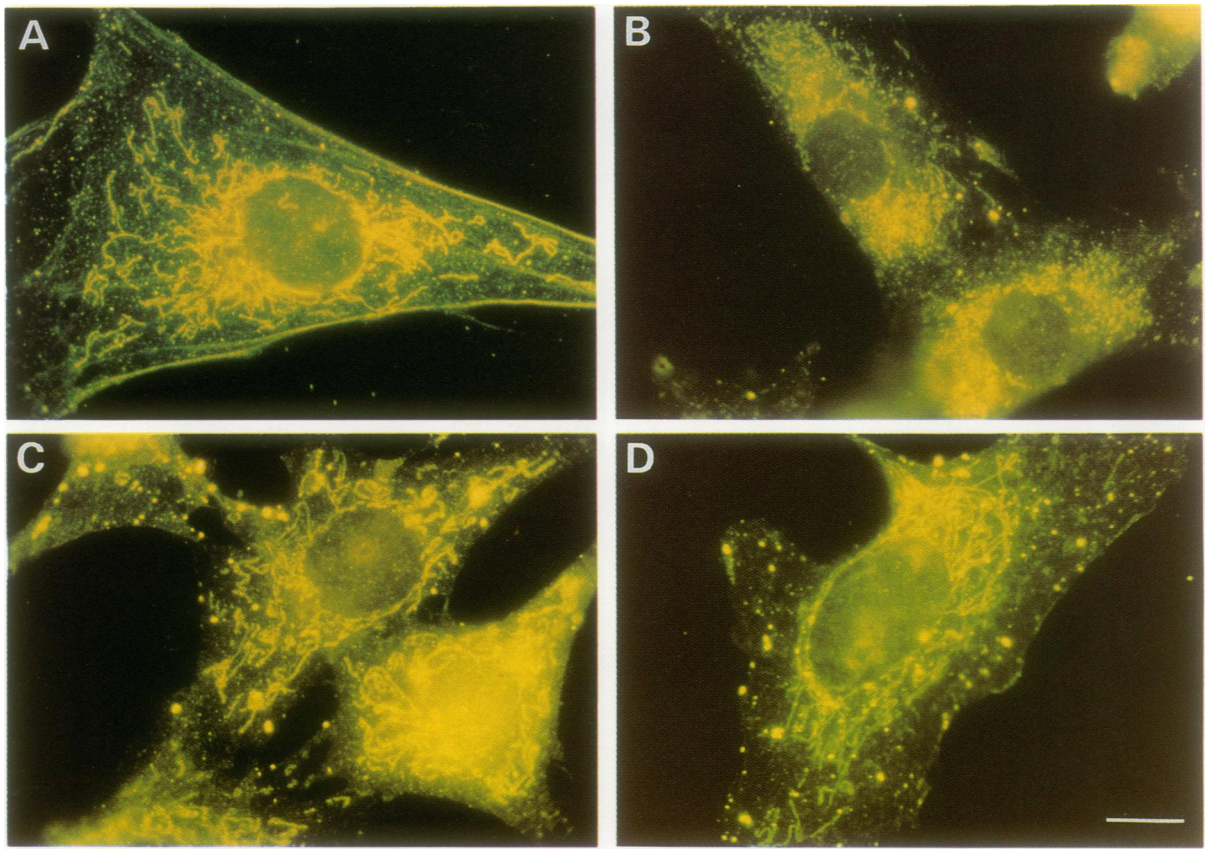


Figure 3. **Detection of $G_{i\alpha}$ within different types of cells.** Indirect immunofluorescence was examined with antiserum 8729 using (A) mouse 3T3 fibroblasts, (B) normal rat kidney fibroblasts, (C) rat C6 glioma cells, and (D) human umbilical vein endothelial cells as described in Figure 2. Bar, 10 μm.

majority of $G_{i\alpha}$ instead cosedimented with alkaline phosphodiesterase I, a marker for plasma membrane (0.25 M sucrose/6% Percoll interface). Resolution of plasma membrane from cosedimenting material such as endoplasmic reticulum and endosomes was not attempted.

Electron microscopy

Immunogold labeling using embedded thin sections provided an alternative means of visualizing $G_{i\alpha}$, one in which the access of antibodies was not dependent on permeabilization with detergents. Mouse 3T3 fibroblasts were fixed in monolayer culture with glutaraldehyde, embedded, and sectioned in planes parallel to the substratum. Sections were incubated with antibodies from 8729 previously adsorbed to 15-nm gold particles, and the distribution of particles was assessed by electron microscopy.

Immunogold labeling of structures within the cytoplasm was clearly evident (Figure 6). Surprisingly, in view of the results obtained by immunofluorescence, the labeled structures resembled lysosomes. There were far fewer gold particles, moreover, than observed at the plasma membrane (i.e., ~15% of total in cross-sections of cells viewed at low magnification; see labeling of plasma membrane below). Gold particles in the lysosomes were distributed throughout the cross-section or at the periphery of these structures, always in a fashion coincident with electron-dense material. Gold particles were occasionally found elsewhere in the cytoplasm but never in large numbers or associated with distinct structures. A lysosomal identity for the labeled cytoplasmic structures was confirmed using antibodies specific for cathepsin B, endolyn 78, and LAMPs-1 and -2 (e.g., Figure 7 for LAMP-1).

Gold particles at the cell periphery were presumed to represent $G_{i\alpha}$ on the inner aspect of the plasma membrane (Figure 8). Phospholipid was not visualized because osmium tetroxide was deleterious to immunolabeling. Gold particles were often found in proximity to filamentous material—probably actin—in apposition to

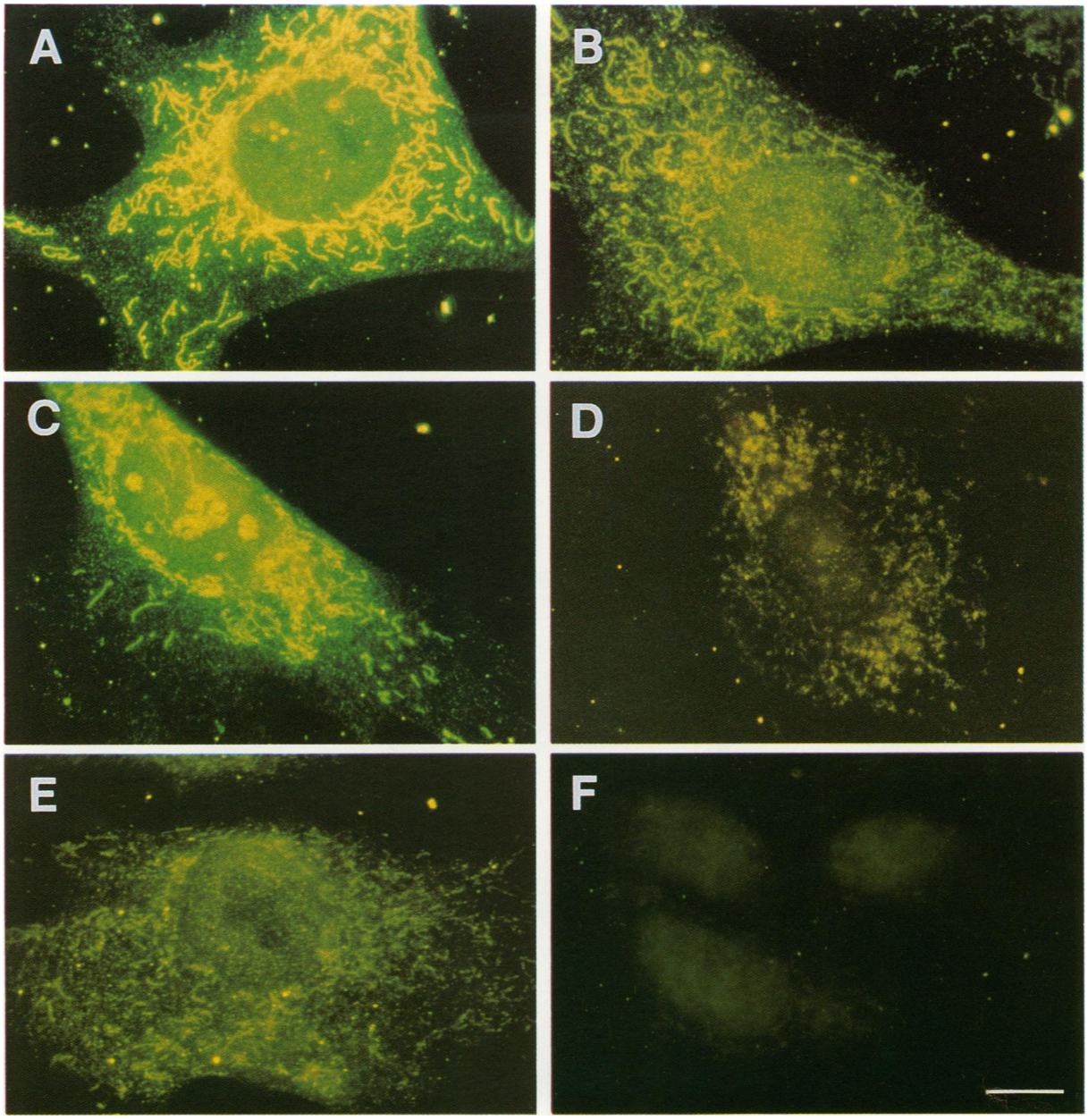


Figure 4. **Detection of $G_{i\alpha}$ with different types of antibodies.** Indirect immunofluorescence is shown for mouse 3T3 fibroblasts with antisera (A) 8729, (B) 5296, (C) 8645, (D) 3646, and (E) 1521, and (F) for human 293 cells with 1521. Bar, 10 μm.

the membrane (Figure 8A). Clusters of three to five particles were commonly noted. Particularly high concentrations of gold particles were present along processes extending from the cell parallel to or transecting the plane of the section (Figure 8B). These structures represent microspikes (Rinnerthaler *et al.,* 1988) whose extensions are active processes in subconfluent cells.

Immunogold labeling of both the cytoplasmic structures and the plasma membrane was specific. No labeling was observed with IgG obtained from preimmune sera or after preincubation of IgG from 8729 with the appropriate peptide. Results were identical when 8645 was substituted for 8729.

Discussion

The subcellular distribution of $G_{i\alpha}$ has an obvious bearing on the ability of this subunit to interact

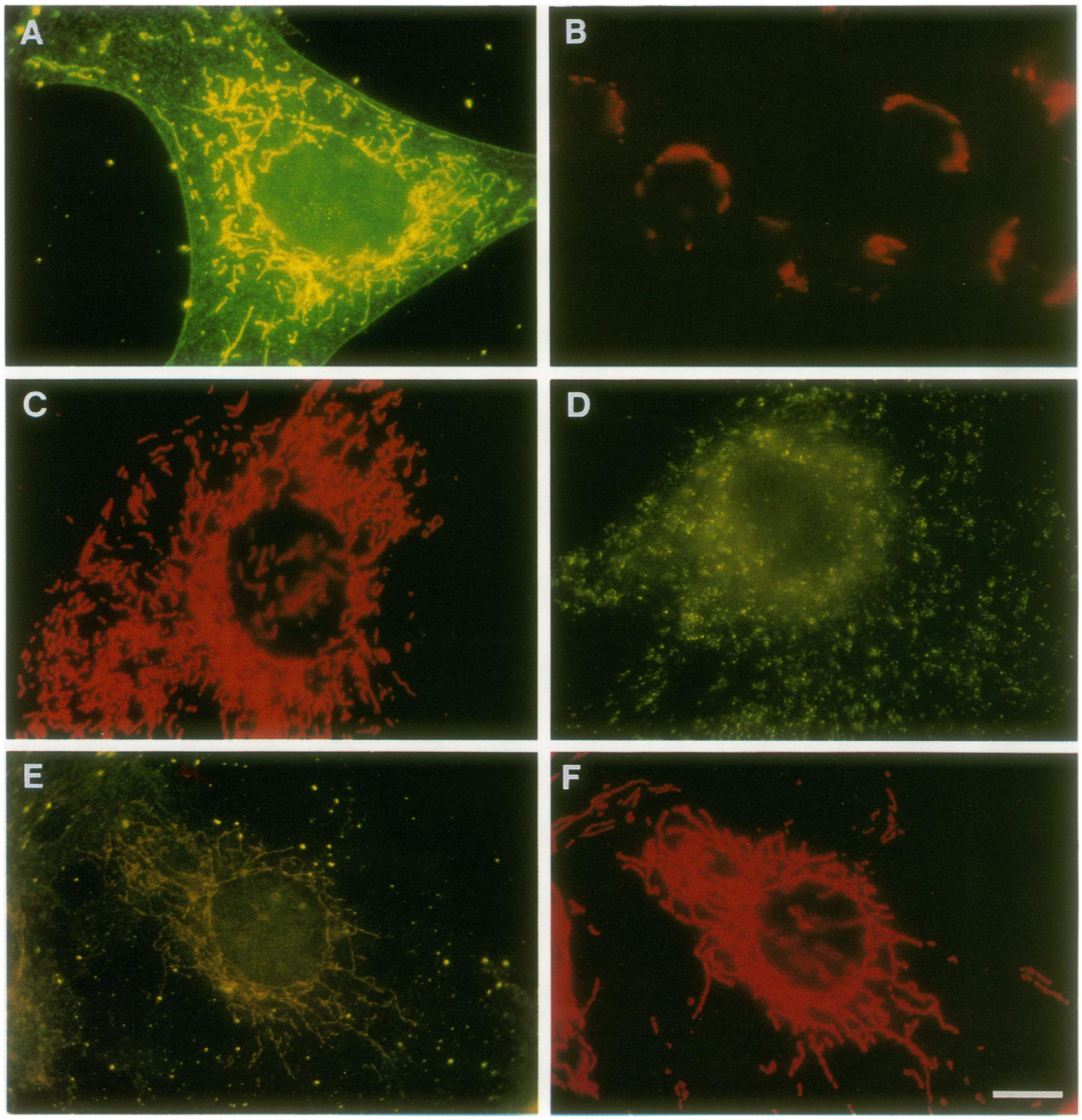


Figure 5. **Distribution of G_{i_α} in relation to various subcellular structures.** Immunofluorescence is shown for (A) mouse 3T3 fibroblasts with antiserum 8729, (B) normal rat kidney cells with a monoclonal antibody to a 160-kDa protein within the medial cisternae of the rat Golgi apparatus, (C) human umbilical vein endothelial cells with a monoclonal antibody toward a 65-kDa human mitochondrial protein, and (D) mouse 3T3 fibroblasts with a monoclonal antibody to LAMP-1. (E and F) represent double-labeling of human umbilical vein endothelial cells with 8729 followed by fluorescein-conjugated goat anti-rabbit IgG, then antibody toward the 65-kDa human mitochondrial protein followed by rhodamine-conjugated sheep anti-mouse IgG.

with receptors and targets and on the potential to serve in still unexplored capacities. In this study, we have examined the distribution of G_{i_α} by means of light and electron microscopy. The results support the proposal that several populations of the subunit indeed exist. One or more populations are associated with cytoplasmic structures whereas another is present at the plasma membrane.

Issues of specificity are critical to any interpretation of immunocytochemical data and such issues were approached initially through im-

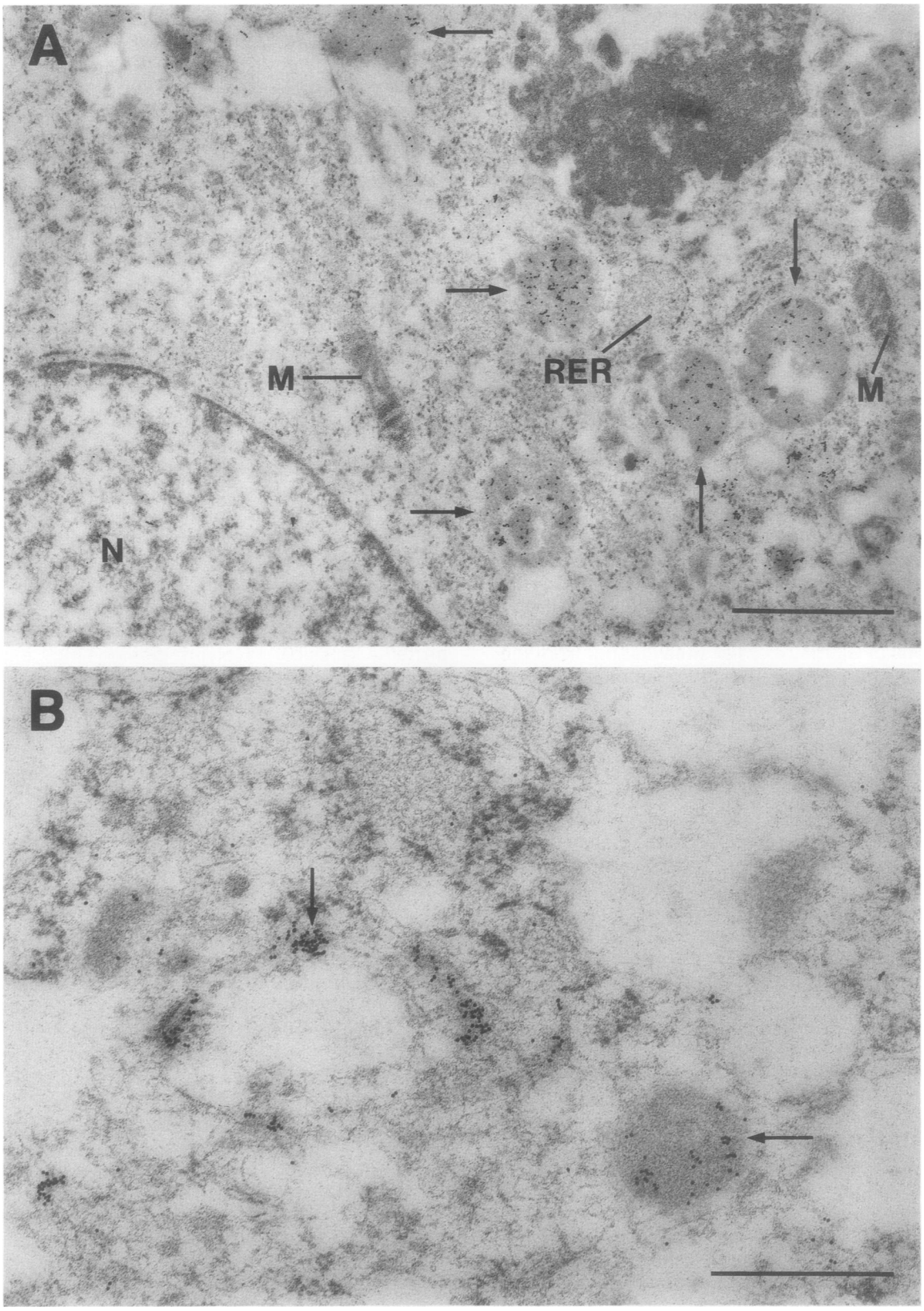
munotransfer blotting. The predominant reactant for all antibodies and cells examined was a 40- to 41-kDa protein(s) present within particulate fractions. As deduced through size and epitopic structure, this protein represents one or a combination of the $G_{i\alpha}$ subtypes. Identification of the protein as $G_{i\alpha}$ is indeed consistent with data obtained previously through immunoprecipitation and isoelectric focusing (Williams *et al.,* 1990). Few other proteins are visualized. Thus, $G_{i\alpha}$ can be recognized at the level of primary structure, and the recognition is specific. Cross-reactivities with proteins considerably less abundant than $G_{i\alpha}$ cannot be precluded by immunoblotting, nor can reactivities beyond primary structure (e.g., originating with KLH). Such reactivities were addressed at the level of immunocytochemistry itself. With regard to the possibility of a small cytosolic (i.e., soluble) population of $G_{i\alpha}$ (Bokoch *et al.,* 1988; Rotrosen *et al.,* 1988), populations representing <5% of the total subunit would not have been detectable. Reactivities with "low-molecular weight" (21–28 kDa) GTP-binding proteins were not anticipated from published sequences and in no instance were such cross-reactivities evident.

Consistent with previous reports that populations of $G_{i\alpha}$ may exist at sites distinct from the plasma membrane, we find by immunofluorescence a substantial amount of the subunit within or attached to structures present in the cytoplasm. Assurances of immunospecificity were provided in part by the similarity in immunofluorescent patterns obtained with antibodies recognizing different epitopes within $G_{i\alpha}$. Thus, although any single antibody may conceivably recognize proteins other than $G_{i\alpha}$, the likelihood that such proteins would contain several epitopes in common is remote. No labeling was observed with preimmune sera or on preincubation of antisera with either peptides used as immunogens or recombinant $G_{i\alpha}$. The latter observations and the similarity in results obtained with affinity-purified antibodies argue against adventitious cross-reactivity at levels beyond primary structure. This argument is bolstered by the inability of KLH alone to block immunofluorescence and the similarity in results obtained with antibodies generated with two substantially different types of antigens, i.e., with KLH-conjugated peptides or purified subunit. Further, human 293 cells, which are deficient in $G_{i\alpha 2}$, provide only a marginal fluorescence with the $G_{i\alpha 2}$-specific antiserum 1521.

The cytoplasmic distribution of $G_{i\alpha}$ as viewed by immunofluorescence appears identical to that of mitochondria, which align in tandem along subsets of microtubules (Goldstein and Korczack, 1981). This distribution suggests an association of $G_{i\alpha}$ either with mitochondria or with other structures similarly utilizing microtubules for transport or determination of shape. Our efforts to demonstrate a direct association with mitochondria have been unsuccessful. Mitochondria purified from 3T3 cells or rat brain (not shown) were found to contain only modest amounts of $G_{i\alpha}$. The lack of $G_{i\alpha}$ in mitochondria is in agreement with the observations of Asano *et al.* (1990), in which $G_{i\alpha}$ present in preparations of mitochondria from rat brain were attributed to contamination with synaptosomal elements. An association between $G_{i\alpha}$ and mitochondria nevertheless remains a formal possibility, in which case attachment would be susceptible to disruption during purification. We have found the immunofluorescent pattern of cytoplasmic $G_{i\alpha}$ to be remarkably sensitive to cell rounding, cell density, and agents that elevate cytoplasmic Ca^{2+} (Lewis, unpublished observations), and such phenomena may conceivably disrupt a tethering of $G_{i\alpha}$ to mitochondria during the course of homogenization and purification. The possibility of a mitochondrial attachment, albeit tenuous, becomes more intriguing as other proteins possibly involved in signaling, e.g., transforming growth factor-β1 (Heine *et al.,* 1991) and Bcl-2 (Hockenbery *et al.,* 1990), are found to be associated predominantly with these organelles.

Other structures aligned along microtubules include tubular lysosomes (Phaire-Washington *et al.,* 1980; Swanson *et al.,* 1987; Heuser, 1989), aspects of the endoplasmic reticulum (Lee and Chen, 1988), and subpopulations of vesicles (Schroer and Kelly, 1985). A lysosomal attachment, although suggested by electron microscopy, is an improbable basis for the predominant immunofluorescence. Immunolabeling of LAMPs-1 and -2 and cathepsin B at the light level, undertaken under conditions identical to that of $G_{i\alpha}$, revealed structures considerably less organized and less tubule-like in appearance. The distribution of lysosomal proteins agreed with that provided by a pH-sensitive vital dye and with literature regarding lysosomes in a variety of cultured cells, including 3T3 fibroblasts (Chen *et al.,* 1985b). The possibility that $G_{i\alpha}$ might interact directly with microtubules is intriguing (Wang *et al.,* 1990). The interaction could occur for $G_{i\alpha}$ attached to organelles or vesicles, as so far discussed, or for the subunit alone.

By electron microscopy and immunogold labeling, $G_{i\alpha}$ is associated with cytoplasmic struc-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tures that are rounded and contain heterogeneous distributions of electron-dense material. As expected, $G_{i\alpha}$ is also associated with the plasma membrane. The cytoplasmic structures are not mitochondria but lysosomes. This identification is consistent both with morphology (Holtzman, 1989) and with labeling obtained for various lysosomal markers. It is probable that the $G_{i\alpha}$ in lysosomes represents a relatively small proportion of subunit present through normal autophagic processes, and by immunofluorescence would be either undetectable or obscured by the more prominent mitochondrial-like labeling. The subunit may be intact or degraded but in either instance recognizable with peptide-directed antibodies. The reason the predominant cytoplasmic population evident by immunofluorescence is not observed by immunogold labeling is unclear. We suspect this population is sensitive to the procedures of fixation and embedding employed, as it is to cell shape and Ca^{2+}. In this regard, our attempts to vary procedures of fixation and embedding have led either to a loss of immunoreactivity altogether or to samples unsuitable for electron microscopy.

The labeling of $G_{i\alpha}$ at the plasma membrane was especially prominent by immunogold labeling. This population of $G_{i\alpha}$ was often viewed as clusters of particles distributed evenly along the periphery. Whether groupings per se represent constrained distributions will require further evaluation. Labeling was particularly concentrated along structures resembling microspikes. Microspikes represent extensions from the cell occurring through polymerization of actin. The pattern of $G_{i\alpha}$ observed at the plasma membrane was remarkably similar to that reported by Péraldi *et al.* (1989) for $G_{o\alpha}$ in ependymal cells. These investigators demonstrated labeling of microvilli on the apical surface of ependymal cells within mouse choroid plexus and of microvilli and kinocilia, respectively, for cultured choroidal and hypothalamic ependymocytes. Even at the level of the plasma membrane, therefore, regulated distributions of G proteins may exist. Support for the possibility that an association of $G_{i\alpha}$ with cytoskeletal elements occurs comes through observations that transduction is often sensitive to disruption of cytoskeletal elements (Kennedy and Insel, 1979; Insel and Koachman, 1982), that a large fraction of $G_{\beta\gamma}$ cofractionates with actin filaments (Carlson *et al.*, 1986), and that certain G_{α} can interact with tubulin (Wang *et al.*, 1990). Conversely, the proximity of $G_{i\alpha}$ to cytoskeletal elements may reflect not constraint of the subunit by these elements but the regulation of the cytoskeleton by $G_{i\alpha}$-controlled second messengers (Wilde *et al.*, 1989).

With all antisera but 3646 and 1518, patterns of fluorescence and gold labeling were probably those established by $G_{i\alpha 2}$. This is the most abundant subtype of $G_{i\alpha}$ (293 cells notwithstanding), exceeding the next by at least fivefold. Antiserum 1521, which is specific for $G_{i\alpha 2}$, indeed provides the same pattern of labeling as do 8729 and 8645, which recognize all three forms. Our results with 3646, a $G_{i\alpha 1}$-specific antiserum, are preliminary, since arguing distinctions among subtypes will require a greater number of subtype-specific antibodies. Some antisera failed to provide signals by fluorescent or gold labeling. Among these was 1398, which recognizes all three subtypes of $G_{i\alpha}$ by immunotransfer blotting. Conceivably, the epitope is occluded by conformation or other proteins.

The distribution of $G_{i\alpha}$ may depend on the degree to which cells assume specialized functions or morphology. The cells examined in this study were largely undifferentiated, representing those most often used as models for growth in monolayer culture. Previous studies, using subcellular fractionation and pertussis toxin-effected radiolabeling, have employed more specialized types of cells. In human neutrophils, $G_{i\alpha}$ is reported to exist within the cytoplasm (perhaps 5%, or greater, of total subunit), in association with specific granules (30%), and at the plasma membrane (~65%) (Bokoch *et al.*, 1988; Rotrosen *et al.*, 1988). In canine pancreas, the majority of $G_{i\alpha}$ is reported to interact with rough endoplasmic reticulum (Audigier *et al.*, 1988), whereas in canine heart a smaller proportion has been identified in sarcoplasmic reticulum (Scherer *et al.*, 1987). Issues related to the recovery and purity of organelles and to quantitative aspects of toxin-labeling are important points to consider. In newly polarized epithelial cells, immunofluorescence using an antibody analogous to 8729 (AS7) (Falloon *et al.*, 1986)

Figure 6. **Immunogold labeling of $G_{i\alpha}$ within cytoplasmic structures.** Immunogold labeling is shown for 3T3 fibroblasts glutaraldehyde-fixed in monolayer culture, sectioned, and exposed to IgG purified from antiserum 8729 and adsorbed to 15-nm gold particles. (A and B) show representative labeled cytoplasmic structures (electron-dense and -lucent) identified by arrows. The nucleus (N), mitochondria (M), and rough endoplasmic reticulum (RER) are also identified. Bars, 1 μm (A) and 0.5 μm (B).

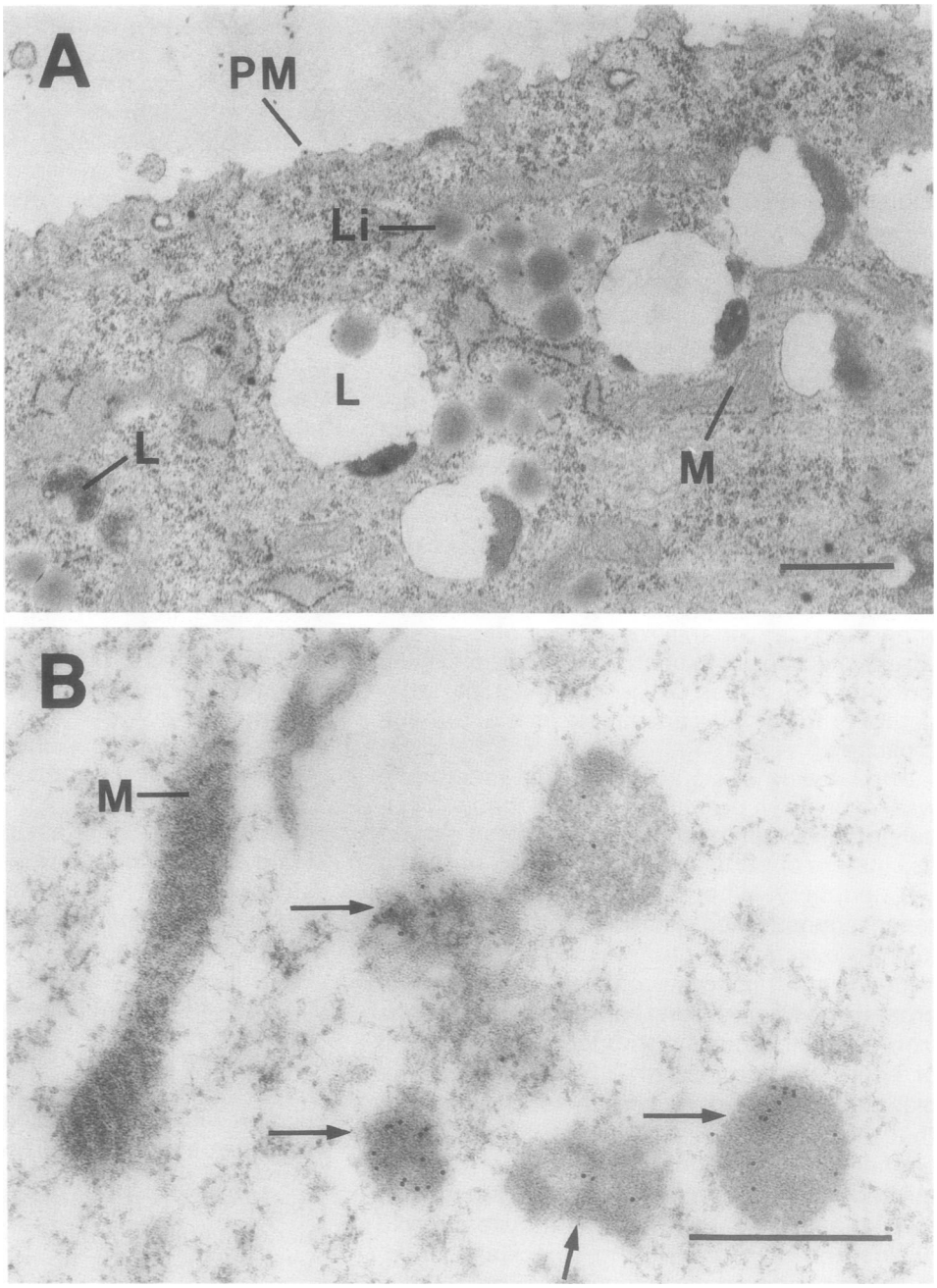


Figure 7. **Identification of lysosomes.** Structures within 3T3 fibroblasts were visualized following (A) postfixation with osmium tetroxide or (B) immunolabeling with antibodies to LAMP-1. (A) Fibroblasts were fixed with glutaraldehyde and postfixed for 1 h with 2% osmium tetroxide in 100 mM sodium cacodylate, pH 7.4. Embedded sections were examined directly (Bar, 1 μm). Structures noted are plasma membrane (PM), a lipid body (Li), lysosomes (L), and a mitochondrion (M). (B) Fibroblasts were fixed with glutaraldehyde, sectioned, and incubated with gold-labeled monoclonal antibodies to LAMP-1 as described previously for 8729. Arrows denote gold-labeled structures (Bar, 0.5 μm).

suggests an association of $G_{i\alpha 2}$ with the basolateral aspect of the plasma membrane (Ercolani *et al.,* 1990). The distribution of this subunit with time after differentiation, however, assumes an appearance almost identical to that found in these studies for fibroblasts and other cells (Holtzman *et al.,* 1991).

Crouch (1990) reported a growth factor-dependent distribution of $G_{i\alpha}$ in 3T3 cells using the AS7 antiserum. In the presence of growth factors such as insulin and epidermal growth factor, the pattern of immunofluorescence was virtually identical to that reported here. This would be expected given the growth of our cells in serum-containing medium. We have been unable to corroborate, however, the reported increase in plasma membrane fluorescence upon serum deprivation or the growth factor-promoted translocation to the nucleus.

The existence of spatially defined populations of $G_{i\alpha}$ has an immediate impact on models of interactions occurring among participants in transduction. The concept that G proteins serve as amplifiers for agonist-occupied receptors (Ross and Gilman, 1980), for example, must be defined in accordance with the population of G proteins to which the receptors gain access. Interactions of receptors with cytoplasmic populations of G_i or with those populations associated with cytoskeletal elements cannot be presumed. Caution must also be extended to interactions among the subunits of G proteins themselves. At the level of G_s and G_i, equilibria among G_α and $G_{\beta\gamma}$ implicit to models of inhibition of adenylyl cyclase (Katada *et al.,* 1984, 1986) may indeed not be realized. G_α are also subject to co- or post-translational forms of modification (Buss *et al.,* 1987; Carlson *et al.,* 1989). Such modifications can assume greater meaning only as the locations of modified subunits are better understood. Myristoylation, for example, is important to the attachment of certain G_α to membranes (Jones *et al.,* 1990; Mumby *et al.,* 1990), but the question remains as to the proportion of G_α modified and the nature of membranes to which attachment occurs. The manner in which patterns of distribution can be regulated in relation to hormonal stimuli or the status of cell movement or differentiation also merits attention. Topological regulation has been characterized to some extent for receptors (Waldo *et al.,* 1983; Sullivan *et al.,* 1984; Jesaitis *et al.,* 1989), but as the emergence of immunocytochemical approaches the characterization can now be extended to G proteins.

Materials and methods

Antibodies

Antisera 1398, 8645, 1013, 8729, 3646, 1521, and 1518 were generated in rabbits using synthetic peptides conjugated to KLH as described previously (Carlson *et al.,* 1989). Specificities for subtypes of $G_{i\alpha}$ were determined through immunotransfer blotting with recombinant forms of the subunit (Carlson *et al.,* 1989; Williams *et al.,* 1990). IgG was purified from sera where indicated by chromatography on protein A-Sepharose (Pierce, Rockford, IL). Purification of peptide-directed antibodies from 8729 was achieved by chromatography on Reacti-Gel (Pierce) to which the peptide used for immunization had been coupled; elution was achieved with 0.1 M glycine, pH 2.9. The G_i used for immunization of rabbits (e.g., in the generation of 5296) was purified as a mixture of G_i and G_o from bovine brain (Sternweis and Robishaw, 1984), whereas that used for immunization of mice was purified from rabbit liver (Bokoch *et al.,* 1984). Monoclonal antibodies used were those recognizing actin (ICN, Costa Mesa, CA), clathrin heavy chain (ICN), a 65-kDa protein specific to human mitochondria (Chemicon, El Segundo, CA), a 160-kDa protein within the medial cisternae of the rat Golgi apparatus (the gift of Drs. Jacqueline and Nicholas Gonatas [Gonatas *et al.,* 1989]), LAMPs-1 and -2 (Developmental Studies Hybridoma Bank, The Johns Hopkins University School of Medicine, Baltimore, MD [Chen *et al.,* 1985a]), and endolyn-78 (the gift of Dr. Melvin Rosenfeld [Croze *et al.,* 1989]). Sheep antibodies to cathepsin B were obtained from Bio Design, Inc (Kennebunkport, ME).

Cell culture

Mouse Swiss 3T3 fibroblasts, normal rat kidney fibroblasts, and rat C6 glioma cells were maintained in Dulbecco's modified Eagles medium (4.5 g glucose/l) containing 10% calf or fetal calf serum. Human umbilical vein endothelial cells were isolated with collagenase and passaged on fibronectin-coated dishes in Medium 199 containing 10% fetal calf serum, 12 units/ml heparin, and endothelial cell growth supplement (Jaffe *et al.,* 1973). Human embryonic 293 kidney fibroblasts were maintained in minimal essential medium containing 10% fetal calf serum. For microscopy, all cells were trypsinized, plated on coverslips, and maintained in supplemented media for 24–48 h before processing. Cells were examined before confluence.

Immunotransfer blotting and analysis for subtypes of $G_{i\alpha}$

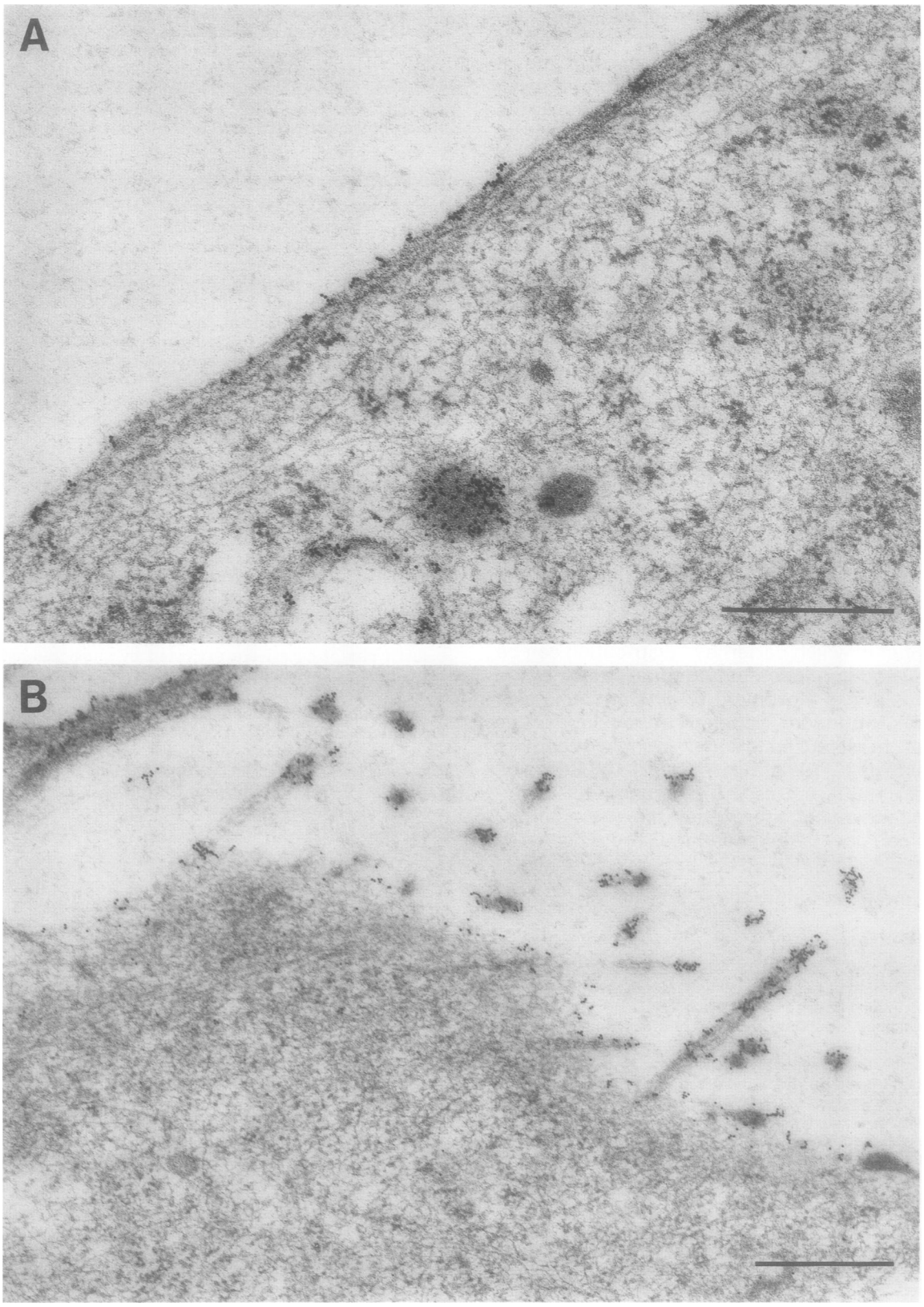
Immunotransfer blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis was accomplished using rabbit antisera at 1:100 dilutions, biotinylated goat anti-rabbit IgG, horseradish peroxidase-conjugated streptavidin (Vector, Burlingame, CA), and H_2O_2 plus 4-chloro-1-naphthol (Carlson *et al.,* 1989). Subtypes of $G_{i\alpha}$ present within individual types of cells were examined by immunotransfer blotting with subtype-specific antibodies using recombinant subunits as standards or by analysis of the isoelectric properties of subunits immunoprecipitated with 1398 from cells incubated with [^{35}S]methionine (Williams *et al.,* 1990). Particulate and soluble fractions of cells were isolated after hypotonic lysis by differential pelleting as described (Woolkalis *et al.,* 1986). Recombinant $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha1}$ were synthesized using plasmid-transformed *Escherichia coli* supplied by Drs. M. Linder and A. Gilman (Linder *et al.,* 1990).

Subcellular fractionation

3T3 cells grown in monolayer culture were detached by scraping, washed in 0.25 M sucrose, and lysed by nitrogen cavitation (30 psi for 15 min) and homogenization as described by Storrie and Madden (1990). The postnuclear supernatant (PNS) was layered above a discontinuous density gradient provided by 6% Percoll, 17% Metrizamide, and 35% Metrizamide and subjected to centrifugation at 50 000 $\times$ *g* for 30 min in a SW-40 rotor. Collected fractions were assayed for alkaline phosphodiesterase I (plasma membrane), β-galactosidase (lysosome), and cytochrome c oxidase (mitochondria), which were found predominantly at the PNS (0.25 M sucrose)/Percoll, Percoll/Metrizamide, and 17/35% Metrizamide interfaces, respectively.

Light microscopy

Cells grown in monolayer culture on glass coverslips (untreated or coated with fibronectin) were usually washed with phosphate-buffered saline (PBS), incubated for 20 min at room temperature with 4% freshly depolymerized paraformaldehyde in 0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8, containing 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetracetic acid and 2 mM $MgCl_2$,


A
B

and permeabilized with 0.2% Triton X-100 or saponin. Fixation with either 95% methanol/5% acetic acid at room temperature or acetone at −20°C was employed as an alternative where described. Washed permeabilized cells were incubated with 10% normal goat serum in PBS for 30 min, then for 45 min at 37°C with either antiserum (or preimmune serum), protein A-purified IgG, or peptide-purified IgG diluted in 10% normal goat serum. Cells were subsequently washed and incubated with goat anti-rabbit IgG conjugated to fluorescein or rhodamine (Cappel, Durham, NC) or biotin (Vector). For immunofluorescence, coverslips were mounted in Fluoromount (Fisher, Pittsburgh, PA) containing 2.5% 1,4-diazabicyclo-(2,2,2) octane (Polysciences, Warrington, PA). Where biotinylated secondary antibodies were used, cells were incubated with horseradish peroxidase-conjugated streptavidin, then with 0.015% H_2O_2 and 0.5 mg/ml diaminobenzidine. Cells were examined by phase-contrast or fluorescence microscopy with a Zeiss III photomicroscope equipped with appropriate filters (Zeiss, Thornwood, NY). Where noted, fluorescence was also analyzed with a MRC 600 confocal microscope (Bio-Rad, Cambridge, MA).

Electron microscopy

Cells grown in monolayer culture on Thermanox coverslips (Nunc) were washed with PBS and fixed for 1 h at room temperature with 1% glutaraldehyde, 0.2% picric acid, and 6 mM eserine in PBS. After dehydration by stepwise additions of ethanol to 75%, cells were infiltrated with a 1:1 mixture of LR White resin and 75% ethanol for 1 h, then resin alone overnight at 4°C, then resin for 2 h at room temperature. Coverslips were placed over gelatin capsules filled with resin and cured at 50°C for 2 d. Sections parallel to the coverslips (60-nm thickness) were mounted on nickel grids. Mounted sections were incubated at room temperature for 1–2 h with 1% ovalbumin or bovine serum albumin (BSA) in PBS and then for 36 h at 4°C with gold-labeled antibodies (see below). Sections were subsequently washed with 10 mM tris(hydroxymethyl)aminomethane-HCl in PBS and deionized water, and stained with neutralized 2% aqueous uranyl acetate for 3 min followed by 1 min with bismuth subnitrate. Sections were observed in a JEOL 100CX electron microscope (Tokyo, Japan).

Gold-labeled IgG was prepared by mixing 150 μg of protein A-purified IgG with 10 ml of a colloidal suspension of 15 nm-diameter gold particles at pH 9.5 (Slot and Geuze, 1985). The colloid was stabilized with 0.1% BSA and the mixture was applied to Ultragel AcA-44 (LKB, Piscataway, NJ) equilibrated with 0.1% BSA in PBS. The colloid eluted in two peaks, the first of which (representing gold aggregates) was discarded. The second was concentrated, sterilized by filtration, and stored for use at 4°C.

Acknowledgments

We acknowledge Dr. Douglas Cines for his gifts of human umbilical vein endothelial cells, Drs. Jacqueline and Nicholas Gonatas for antibodies recognizing MP 160, Dr. Melvin Rosenfeld for antibodies to endolyn-78, and Dr. John Murray for discussions and use of the confocal microscope. Marie Elena Hudzicki and Neelima Shah provided expert technical assistance. These studies were supported by National Institutes of Health grants GM34781, DK19525, and DK28143. D.R.M. is an Established Investigator of the American Heart Association. M.J.W. is supported by awards from the Southeastern Pennsylvania American Heart Association and the W.W. Smith Charitable Trust.

Received: March 1, 1991.
Revised and accepted: September 9, 1991.

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Figure 8. **Immunogold labeling of $G_{i\alpha}$ at the cell periphery.** Immunogold labeling was accomplished as described in Figure 6, with the cell periphery alone shown. (A) Labeling is evident as evenly distributed clusters of particles in apposition to filamentous material. (B) Labeling of microspikes transecting or extending within the plane of the section is evident. Bars, 0.5 μm.

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