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Differential Localization of G-proteins, Gαo and Gαi-1, -2, and -3, in Polarized Epithelial MDCK Cells

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MDCK cells were stably transfected with rat Gαo cDNA and confluent polarized monolayers were analyzed by immunocytochemistry to compare the intracellular targeting of Gαo with the localization of the endogenously expressed Gαi subunits. Immunofluorescence confocal microscopy showed that Gαo is targeted strictly to the lateral membrane. Immunolocalization of Gαi-1, -2, and -3 showed that Gαi-1 and -2 are confined to the cytoplasm and Gαi-3 is found on the lateral membrane, in the cytoplasm, and faintly on the apical surface of these cells. Thus, the different pertussis toxin-sensitive G-proteins are differentially localized in polarized epithelial cells. © 1997 Academic Press

Epithelial cells are able to form a barrier between two compartments because they establish tight junctions which seal the intercellular spaces between cells (1). These junctions are formed where the apical plasma membrane bordering the lumen and the basolateral membrane join. These two membranes have distinct lipid and protein compositions allowing the cell layer to carry out transepithelial transport. Studies on protein trafficking have been carried out with homogeneous cell lines because native epithelia have multiple cell types. LLC-porcine kidney (LLC-PK1) cells and Madin-Darby canine kidney (MDCK) cells are frequently used for polarity studies because they maintain tight junctions, carry out vectorial ion transport, and maintain the polarized distribution of lipids and proteins that kidney epithelium possess *in vivo* (1).

Heterotrimeric (α,β,γ) guanine nucleotide binding proteins (G-proteins) are necessary intermediaries in a diverse number of signal transduction pathways involving seven-transmembrane receptors in epithelia. Agonist-bound receptors catalyze the exchange of GTP for GDP on the α-subunit, promoting dissociation from the $\beta\gamma$ complex and allowing interaction with effector proteins. The intrinsic GTPase activity of the α-subunit returns it to the GDP-bound state, capable of binding the $\beta\gamma$ complex again. It is largely the GTP-binding α-subunit which confers specificity for the receptor-effector system, although the $\beta\gamma$ complex is also involved in signaling and possibly in membrane localization. More than 20 different α-subunit cDNA's have been cloned and classified into four major categories (2, 3). One class of G-proteins consisting of Gαi-1,-2, and-3, and two subtypes of Gαo, is susceptible to covalent modification by pertussis toxin which interferes with interactions between G-protein alpha subunits and their receptors (4). The three subtypes of Gαi proteins were identified originally by their involvement in the inhibition of adenylyl cyclase. In addition they can couple to K^+ channels and can activate phospholipase C. The two subtypes of Gαo, which arise by differential splicing of a single gene, are the most abundant G-proteins in the brain and are involved in regulating the activity of K^+ and Ca^{+2} ion channels and can also inhibit adenylyl cyclase activity (5, 6).

Gαi subunits have been found on the plasma membrane and on membranes of several organelles such as the endoplasmic reticulum, Golgi complex, and the nucleus (7). More specifically, Gαi subunits are found segregated into specific subdomains of the plasma membrane. In LLC-PK1 cells Gαi-2 is restricted to the basolateral membrane where it is most likely involved in adenylyl cyclase inhibition. When the Gαi-2 subunit is overexpressed in LLC-PK1 cells it is also targeted strictly to the basolateral membrane (8). Gαi-3 is confined mostly to the perinuclear region in a Golgi-like distribution where it is known to be involved in regulating trafficking through the secretory pathway (9). It is also found at low levels on the apical surface (8). When this protein is overexpressed threefold it is still targeted predominantly to the Golgi (9). The specificity of these G-proteins for their effectors is most likely accomplished by their segregation to different cellular compartments.

Abbreviations used: G-protein, guanine nucleotide-binding regulatory protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAChR, muscarinic acetylcholine receptor.

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Differentiated neurons exhibit distinct polarization in the functional differences they maintain between axonal and dendrital processes. Polarized epithelial cells in many cases can serve as a model for the neuronal targeting of proteins. Glycosyl phosphatidylinositol (GPI)-anchored proteins are preferentially expressed on axons (10) and the apical surfaces of epithelial cells (11, 12). The transferrin receptor, targeted to the basolateral surface of epithelial cells, is found in the dendrites and cell bodies of neurons (13). When combined with the findings that viral glycoproteins are targeted in a polarized manner in both neuronal and epithelial cells, it has been proposed that these two cell types may share molecular mechanisms of sorting and that axonal/apical and dendritic/basolateral membranes may be analogous domains (14). Because several results contradict this, the targeting of a variety of proteins is being pursued in several cell types. Go is the predominant heterotrimeric G-protein in neuronal tissue where it serves as an intermediary in a variety of signal transduction pathways. We chose to explore the targeting of this protein in the MDCK epithelial cell line which frequently serves as a model for neuronal protein sorting.

The polarized epithelial MDCK cell line has been used to examine the targeting of a large number of both endogenous and transfected G-protein-coupled receptors (15, 16, 17). The localization of G-protein subunits themselves in MDCK cells have not, however, been extensively characterized. In this study we have determined the subcellular localization of endogenously expressed Gαi-1,-2, and-3 in MDCK cells. Because Gαo is the most abundant G-protein alpha subunit in the brain and has been implicated in a number of neuronal signaling pathways, we have also determined its localization in MDCK cells stably expressing Gαo. We find that Gαi-1 and -2 are located in the cytoplasm of these cells and Gαi-3 is found in the cytoplasm, on lateral membranes, and faintly on the apical surface. MDCK cells stably transfected with rat Gαo cDNA target this protein strictly to the lateral membrane.

MATERIALS AND METHODS

Materials

The rat Gαo clone (18), a gift from R. Reed, was expressed in the vector pCD-PS (19). The pK-neo plasmid containing the neomycin phosphotransferase gene was provided by G. S. McKnight (University of Washington). Go, partially purified from bovine brain, and an affinity purified antibody raised against the C-terminal decapeptide of Gαo were gifts from A. Spiegel (National Institutes of Health) and were used for immunoblot analyses of the cell lines. Antibody EC, also a gift from A. Spiegel, was raised against the C-terminal decapeptide of Gαi-3 and recognizes Gαi-3 and Gαo (20). Monoclonal antibodies R4, L5, and 2A recognize Gαi1, Gαi2, and Gαo, respectively (21) and were generously provided by Richard S. Jope, University of Alabama at Birmingham. Goat anti-rabbit and anti-mouse IgG conjugated to FITC were obtained from Cappell. G418 was purchased from Sigma and Lipofectin from GIBCO BRL.

Cell Culture

MDCK (strain I) cells, obtained from America Type Culture Collection, were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin G (100 U/ml)/streptomycin sulfate (0.1 mg/ml; Apothecan). Cells were maintained in a 10% CO_2 atmosphere at 37°C.

Generation of Stable Cell Lines

MDCK cells cultured on 10 cm plates were co-transfected using Lipofectin (Gibco/BRL) with 40 μg of the expression plasmid pCD-PS containing the rat Gαo cDNA and 4 μg of pK-neo containing the neomycin phosphotransferase gene. Colonies resistant to G418 (1.2 mg/ml) were picked 17 days later. Two lines out of the seven resistant to G418 expressed the Gαo gene as identified by immunoblots probed with the affinity-purified polyclonal antibody to Gαo.

Immunoblot Analyses

Crude cell membranes were prepared as described in (22) except that cells were homogenized by hand with 20 strokes of a ground glass homogenizer. Samples (5-15 μg protein) were loaded on a 10% acrylamide/0.16% bis-acrylamide SDS gel. Partially purified Go (2 μg) from bovine brain was used as a control. Proteins were transblotted by wet transfer onto nitrocellulose and incubated with a 1/400 dilution of the affinity-purified anti-Gαo polyclonal antibody. Labeled bands were detected with goat anti-rabbit antibody conjugated to alkaline phosphatase (22).

Immunofluorescent Labeling

Cells were grown on autoclaved glass slides (VWR) and processed 3 to 5 days post-confluency to ensure that polarity was established. Cells were rinsed with PBS and fixed 30 minutes in 4% formaldehyde in PBS containing 4% sucrose at room temperature. Cells were permeabilized with Triton X-100 (0.25% in PBS) for 5 minutes, blocked with 10% BSA at room temperature for 2 hours, and incubated overnight at 4°C with primary antibodies used at the following dilutions: Gαi-1 (R4) 1/250, Gαi-2 (L5) 1/250, Gαi-3 (EC) 1/500, and Gαo (2A) 1/250. The following day cells were rinsed with PBS and incubated with fluorescein-conjugated goat anti-rabbit/mouse IgG (1/400) for 2 hours at room temperature. Stained cells were rinsed again in PBS, coverslipped with Vectashield (Vector), and viewed by conventional epifluorescence on a Leitz Dialux 20 microscope or by confocal imaging on a Bio-Rad 600 MRC system.

RESULTS

Immunoblot analysis (Figure 1) with an affinity purified polyclonal antibody raised against the C-terminal decapeptide of Gαo was used to identify the MDCK line stably transfected with Gαo (lane 1). Partially purified Go derived from bovine brain (22) was used to identify the appropriate immunoreactive band at 39 kDa (lane 3). Crude cell membranes from a transfected but non-Gαo expressing cell line showed no band at 39kDa (lane 2). While this polyclonal antibody yielded consistent specific labeling on immunoblots, it gave inconsistent results when used for immunocytochemical analyses. All light and confocal microscopy results shown here were obtained using the monoclonal antibody (2A) generated against Gαo (21).

Immunostaining of MDCK cells stably transfected with Gαo shows a predominantly lateral signal as evi-

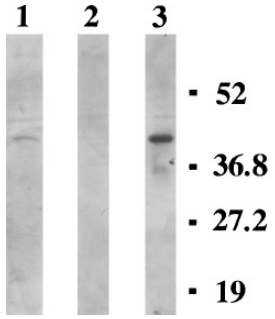

FIG. 1. Immunoblot analysis of MDCK cell membranes used to identify cell lines stably expressing Gαo. Crude cell membranes were analyzed by SDS-PAGE and the transblot was incubated with affinity-purified polyclonal antisera recognizing Gαo. Proteins were visualized using the alkaline phosphatase detection method (see Materials and Methods for additional details). Lane 1, membranes from cells stably transfected with rat Gαo cDNA. Lane 2, membranes from untransfected cells. Lane 3, 2 μg partially purified bovine Gαo.

dent by the characteristic cobblestone pattern (Figure 2A). Untransfected MDCK cells show virtually no signal or a slight cytoplasmic labeling when photographed at the same exposure settings (Figure 2B).

Localization of Giα-1,-2, and-3 by immunostaining using monoclonal antibodies R4 and L5 (21) and polyclonal antibody EC (20), respectively, is shown in Figure 3A-C. The targeting of these subunits was identical in cells stably transfected with Gαo and in untransfected cells (data not shown). Confocal images show that Gαi-1 and -2 are confined to the cytoplasm. Gαi-3 shows strong labeling in both the cytoplasm and along the lateral edges of the cells. These results differ somewhat from those in LLC-PK1 cells where Gαi-2 is confined to the basolateral membrane (8) and Gαi-3 is found predominantly in the cytoplasm associated with Golgi bodies (9), but not on the lateral membranes. MDCK cells stably transfected with Gαo sort this protein exclusively to the lateral membrane (Figure 3D).

A set of confocal images collected from the apical to basolateral surfaces at 1 μm intervals ("z-series") is shown for each antibody in Figure 4. These images corroborate the localizations shown in the single midplane photos of Figure 3 and demonstrate that little or no labeling is seen in the apical and basal surfaces with antibodies against Gαi-1,-2, and Gαo. The first three images of the Gαi-3 series show a low level of apical localization. LLC-PK1 cells also show a low level of Gαi-3 labeling on the apical surface (9).

A series of images (xz-sections) were collected on the confocal microscope at 0.17 μm intervals from apical to basolateral membranes (inset, Figure 5). The staining of lateral membranes with the anti-Gαo monoclonal antibody is prominent in the columnar signal obtained. Xz-sections for the other three antibodies showed labeling in a smear surrounding the nucleus (data not shown).

DISCUSSION

We have shown here that Gαi-1 and -2 are found in the cytoplasm of the polarized epithelial canine kidney cell line, MDCK (strain I). Gαi-3 is localized predominantly to both the cytoplasm and the lateral edges of these cells. A confocal "z-series" of images at 1 μm intervals also shows faint localization of Gαi-3 on the apical surface. MDCK cells stably transfected with rat Gαo cDNA target the expressed protein strictly to the lateral membrane.

The localizations reported here differ somewhat from those reported for the porcine kidney epithelial cell line, LLC-PK1. Gαi-1 was not detected in these cells by immunoblot and RNA blot analyses and Gαi-2 was found mainly on the basolateral membranes (8). Gαi-3 was localized predominantly in a Golgi-like pattern and faintly on the apical surface of some cells (8, 9); it was not found associated with the basolateral membrane in LLC-PK1 cells. While this manuscript was in preparation, results were published showing the involvement of G-proteins in tight junction biosynthe-

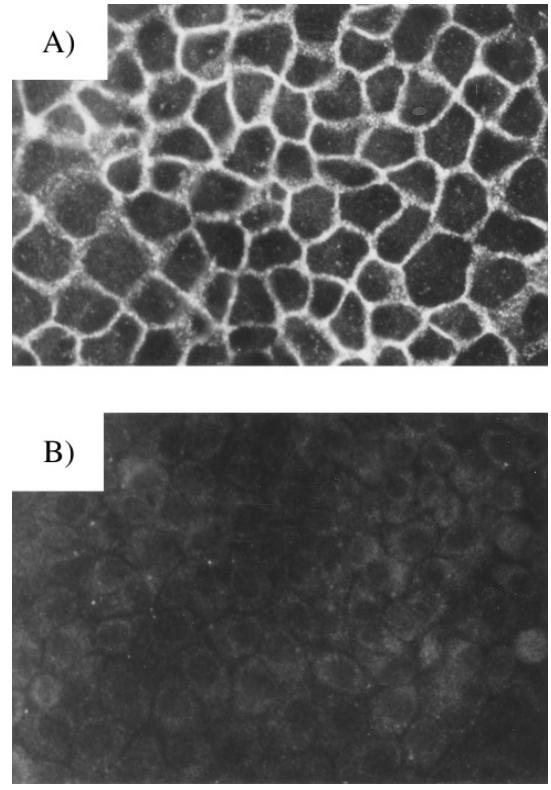

FIG. 2. Immunofluorescent labeling of MDCK cells stably transfected with Gαo shows the cobblestone pattern typical of lateral localization. Confluent monolayers of cells were incubated with monoclonal antibody 2A which recognizes Gαo. Cells were exposed to a fluorescein-conjugated secondary antibody and viewed by confocal imaging (see Material and Methods). A, MDCK cells stably transfected with rat Gαo cDNA. B, untransfected MDCK cells photographed at the same exposure settings.

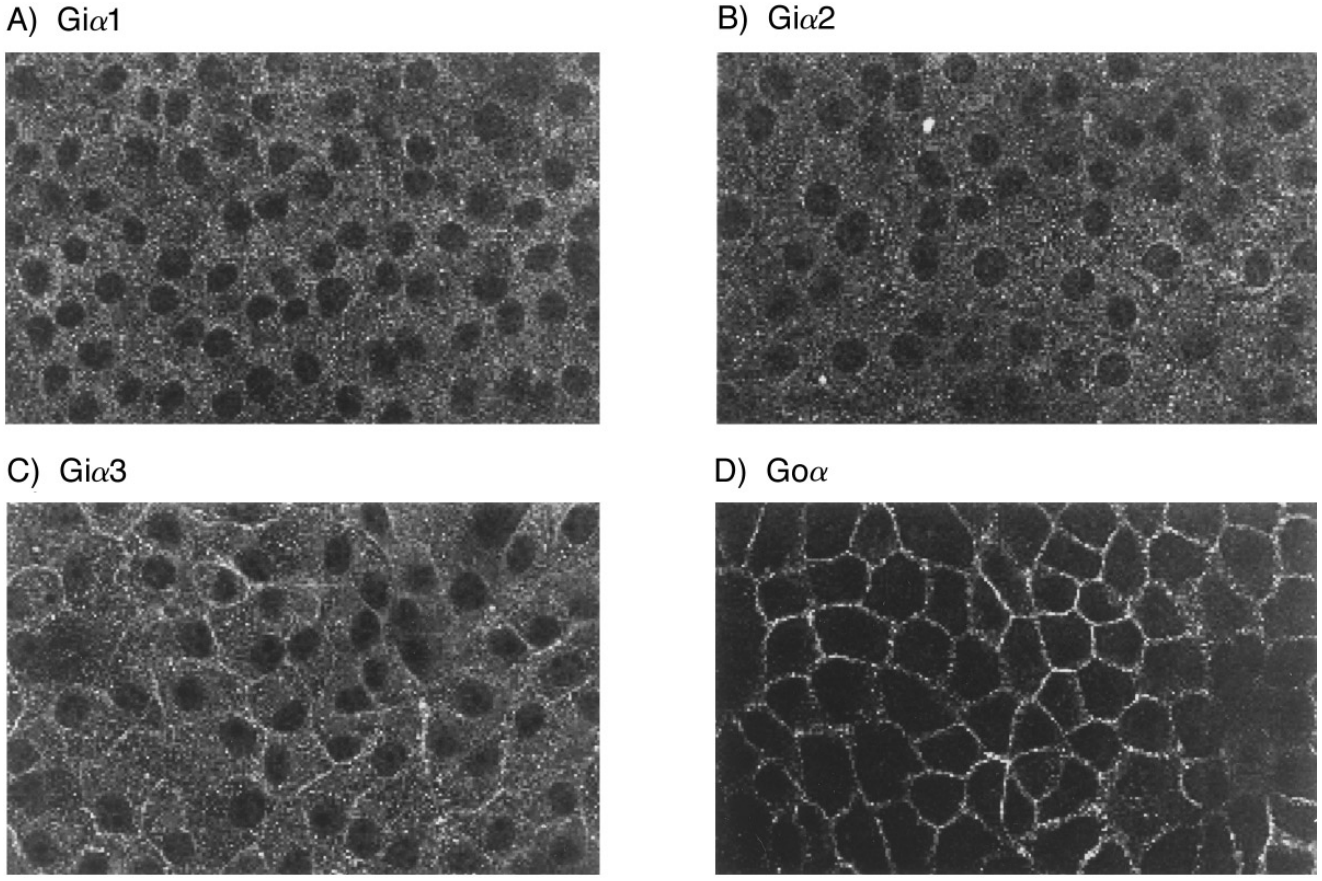

FIG. 3. Immunolocalization of Gαi-1, Gαi-2, Gαi-3, and Gαo in MDCK cells. Confluent monolayers of cells were incubated with anti-G-protein antibodies, then exposed to fluorescein-conjugated secondary antibody and viewed by confocal microscopy. A, monoclonal antibody R4, recognizing Gαi-1, shows cytoplasmic labeling. B, monoclonal antibody L5, recognizing Gαi-2, also shows cytoplasmic staining. C, polyclonal antibody EC, recognizing Gαi-3, labels both the cytoplasm and lateral membranes of the cells. D, monoclonal 2A, recognizing Gαo, labels only the lateral cell membranes.

sis (23). Our results concur with theirs regarding the localization of Gαo in MDCK cells transfected with rat Gαo cDNA. However, they report that Gαi-2 is targeted to the lateral membrane with some intracellular staining in MDCK cells. Using the monoclonal antibody specific for Gαi-2 we see only cytoplasmic labeling in these cells.

Because Gαo is the major G-protein alpha subunit in the brain, knowledge of the mechanisms involved in its action and cellular localization is important in understanding its physiological functions. Go is found in differentiating neurons extending neurites and has been implicated in initiation and maintenance of neurite outgrowth (24). GAP-43 (growth-associated protein), also called neuromodulin, is an intracellular protein that colocalizes with Gαo throughout the nervous system (25). In solution GAP-43 stimulates GTP binding to Gαo in a manner that is insensitive to pertussis toxin (26), indicating that Go can be activated by proteins other than seven transmembrane receptors.

Gαo can also couple receptors to the inhibition of adenylyl cyclase activity and the regulation of ion channels. For example, Go can couple muscarinic receptors to both inhibition of adenylyl cyclase (27) and inactivation of calcium channels (6).

Go is involved in many aspects of behavior in C. elegans (28, 29). It is found in neurons, in the vulva and uterine muscles of hermaphrodites, and in the diagonal muscles of the male. Mutations resulting in reduced function of Go cause hyperactive movement, premature egg-laying, and impotence. Constitutively active mutants of Go cause lethargy, retention of eggs, and a reduced ability of the male to mate.

Several lines of evidence suggest the importance of Go in Alzheimer's disease. The wild type amyloid precursor protein (APP_{695}) colocalizes with Gαo in growth cones and neuronal presynapses and is able to form a complex through its cytoplasmic domain with Gαo that is disrupted by pertussis toxin treatment. The APP_{695} cytoplasmic peptide corresponding to His 657-Lys 676 can activate Gαo *in vitro* (30) and *in vivo* (31). Coexpression of Gαo with APP containing mutations which

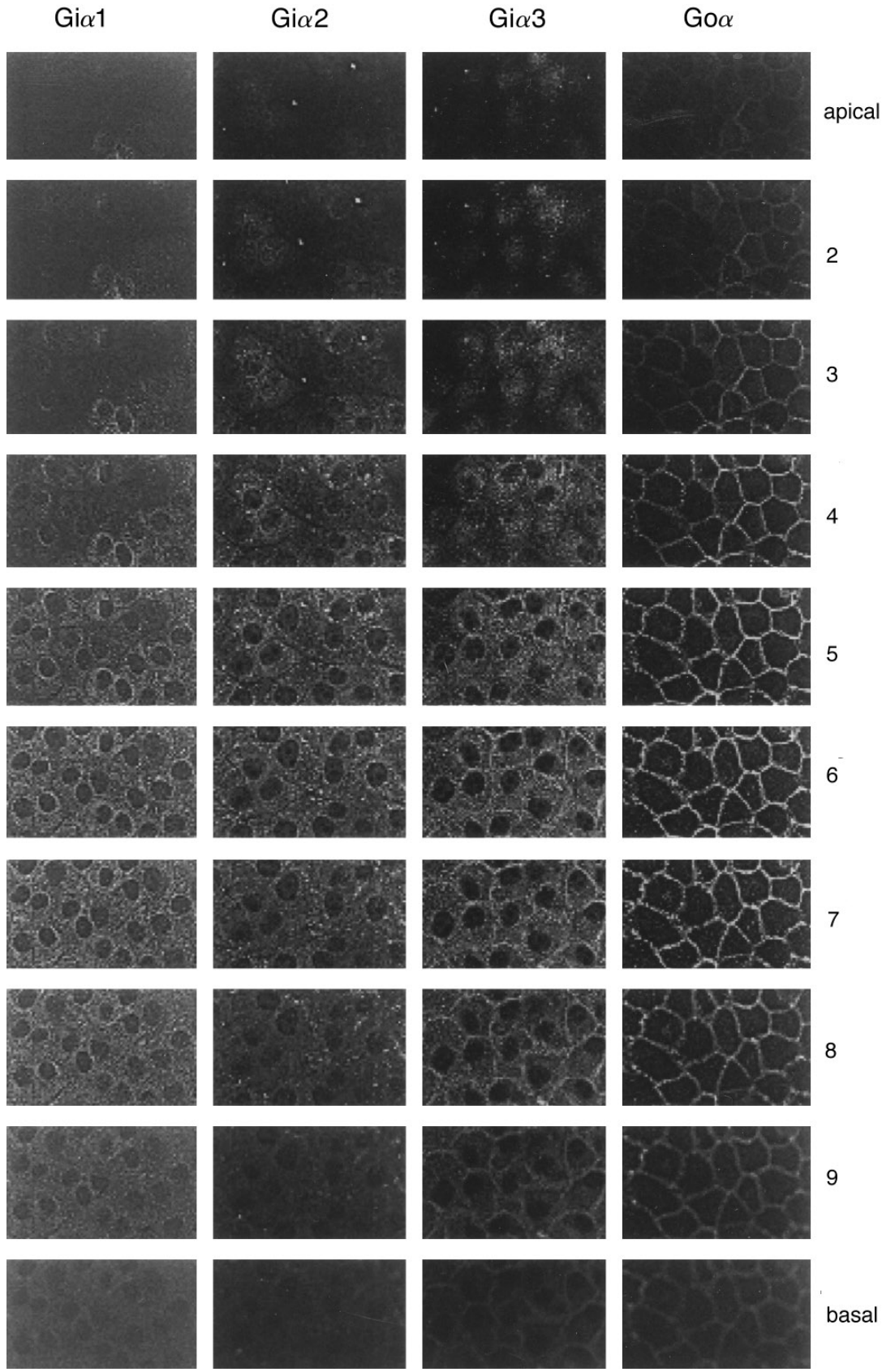

FIG. 4. Sets of images ("z-series") taken at 1 μm intervals from apical (image 1) to basal (image 10) membranes by confocal microscopy of confluent MDCK cells stably transfected with rat Gαo cDNA. Localizations described in the legend to Fig. 3 are evident throughout the cell. In addition, however, Gαi-3 shows a faint apical signal (images 1-3).

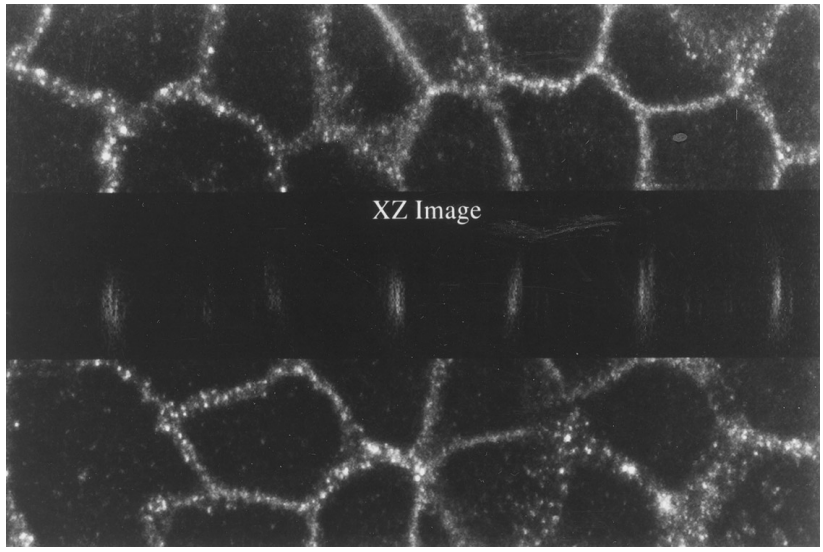

FIG. 5. Localization of Gαo in the xz plane (inset) in a confluent monolayer of MDCK cells stably transfected with Gαo. Confocal images were collected at 0.17 μm intervals from the apical to basolateral surface along the z-axis at the cross-section of the inset. The data are presented as if one were viewing the monolayer from the side and show that localization is strictly limited to the lateral membranes.

cause Alzheimer's disease will induce apoptotic cell death and these effects are inhibited by pertussis toxin treatment and by dominant negative Gαo (32). In addition the mutant APP can activate Go in the absence of ligand (32). This result suggests that APP_{695} could be a Go-linked receptor.

Gαo has been implicated as a regulator of vesicular traffic in a variety of systems. Gαo1 is localized to intracellular vesicular membranes in C6 glioma cells. When these cells stably over-express Gαo1, there is an increase in the secretion of the protease inhibitor, protease nexin-1 (PN-1), a glia-derived neurite-promoting factor. Activators of Go-proteins cause an increase in PN-1 secretion in both parental and transfected cells lines (33). Go is also found associated with membranes of the trans-Golgi network in PC-12 cells (34).

The subcellular localization of G-proteins and the mechanisms responsible for targeting members of the Gi/Go subfamily may be important determinants of differences in their cellular functions. The differential subcellular localization of these proteins shown in this study demonstrates an additional potential layer of complexity in specifying G-protein signal transduction pathways.

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REFERENCES

1. Ojakian, G. K., and Schwimmer, R. (1988) *J. Cell Biol.* **107,** 2377–2387.
2. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) *Science* **252,** 802–808.
3. Spiegel, A. M., Shenker, A., and Weinstein, L. S. (1992) *Endocr. Rev.* **13,** 536–565.
4. Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R. D., Manclark, C. R., Stryer, L., and Bourne, H. R. (1984) *J. Biol. Chem.* **259,** 23–26.
5. Birnbaumer, L., Abramowitz, J., and Brown, A. M. (1990) *Biochem. Biophys. Acta* **1031,** 163–224.
6. Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G., and Wittig, B. (1991) *Nature* **353,** 43–48.
7. de Almeida, J. B., Holtzman, E. J., Peters, P., Ercolani, L., Ausiello, D. A., and Stow, J. L. (1994) *J. Cell. Sci.* **107,** 507–515.
8. Ercolani, L., Stow, J. L., Boyle, J. F., Holtzman, E. J., Lin, H., Grove, J. R., and Ausiello, D. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4635–4639.
9. Stow, J. L., de Almeida, J. B., Narula, N., Holtzmann, E. J., Ercolani, L., and Ausiello, D. A. (1991) *J. Cell Biol.* **114,** 1113–1124.
10. Dotti, C. G., and Simons, K. (1990) *Cell* **62,** 63–72.
11. Powell, S. K., Lisanti, M. P., and Rodriguez-Boulan, E. (1991) *Am. J. Physiol.* **260,** C715–C720.
12. Wilson, J. M., Fasel, N., and Kraehenbuhl, J.-P. (1990) *J. Cell Sci.* **96,** 143–149.
13. Cameron, P. L., Sudhof, T. C., Jahn, R., and DeCamilli, P. (1991) *J. Cell Biol.* **115,** 151–164.
14. Rodriguez-Boulan, E., and Powell, S. K. (1992) *Annu. Rev. Cell Biol.* **8,** 395–427.
15. Mohuczy-Dominiak, D., and Garg, L. C. (1992) *J. Am. Soc. Nephrol.* **3,** 170–181.

16. Keefer, J. R., and Limbird, L. E. (1993) *J. Biol. Chem.* **268,** 11340–11347.
17. Saunders, C., Keefer, J. R., Kennedy, A. P., Wells, J. N., and Limbird, L. E. (1996) *J. Biol. Chem.* **271,** 995–1002.
18. Jones, D. T., and Reed, R. R. (1987) *J. Biol. Chem.* **262,** 14241–14249.
19. Bonner, T. I., Young, A. C., Brann, M. R., and Buckley, N. J. (1988) *Neuron* **1,** 403–410.
20. Simonds, W. F., Collins, R. M., Spiegel, A. M., and Brann, M. R. (1989) *Biochem. Biophys. Res. Commun.* **164,** 46–53.
21. Li, X., Mumby, S. M., Greenwood, A., and Jope, R. S. (1995) *J. Neurochem.* **64,** 1107–1117.
22. Luetje, C. W., Gierschik, P., Milligan, G., Unson, C., Spiegel, A., and Nathanson, N. M. (1987) *Biochemistry* **26,** 4876–4884.
23. Denker, B. M., Saha, C., Khawaja, S., and Nigam, S. K. (1996) *J. Biol. Chem.* **271,** 5750–25753.
24. Strittmatter, S. M., Fishman, M. C., and Zhu, X.-P. (1994) *J. Neurosci.* **14,** 2327–2338.
25. Schmidt, C. J., Zubiaur, M., Valenzuela, D., Neer, E. J., and Drager, U. C. (1994) *J. Neurosci. Res.* **38,** 182–187.
26. Strittmatter, S. M., Valenzuela, D., Sudo, Y., Linder, M. E., and Fishman, M. C. (1991) *J. Biol. Chem.* **266,** 22465–22471.
27. Migeon, J. C., Thomas, S. L., and Nathanson, N. M. (1994) *J. Biol. Chem.* **269,** 29146–29152.
28. Mendel, J. E., Korswagen, H. C., Liu, K. S., Hajdu-Cronin, Y. M., Simon, M. I., Plasterk, R. H. A., and Sternber, P. W. (1995) *Science* **267,** 1652–1655.
29. Segalat, L., Elkes, D. A., and Kaplan, J. M. (1995) *Science* **267,** 1648–1651.
30. Nishimoto, I., Okamoto, T., Matsuura, Y., Takahashi, S., Okamoto, T., Murayama, Y., and Ogata, E. (1993) *Nature* **362,** 75–79.
31. Lang, J., Nishimoto, I., Okamoto, T., Regazzi, R., Kiraly, C., Weller, U., and Wollheim, C. (1995) *EMBO J.* **15,** 3635–3644.
32. Okamoto, T., Takeda, S., Giambarella, U., Murayama, Y., Matsui, T., Katada, T., Matsuura, Y., and Nishimoto, I. (1996) *EMBO J.* **15,** 3769–3777.
33. Lagriffoul, A., Charpentier, N., Carrette, J., Tougard, C., Bockaert, J., and Homburger, V. (1996) *J. Biol. Chem.* **271,** 31508–31516.
34. Leyte, A., Barr, F. A., Kehlenbach, R. H., and Huttner, W. B. (1992) *EMBO J.* **11,** 4795–4804.