Targeting of chimeric Gα$_i$ proteins to specific membrane domains

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SUMMARY

Heterotrimeric guanine nucleotide-regulatory (G) proteins are associated with a variety of intracellular membranes and specific plasma membrane domains. In polarized epithelial LLC-PK$_1$ cells we have shown previously that endogenous Gα$_{i-2}$ is localized on the basolateral plasma membrane, whereas Gα$_{i-3}$ is localized on Golgi membranes. The targeting of these highly homologous Gα$_i$ proteins to distinct membrane domains was studied by the transfection and expression of chimeric Gα$_i$ proteins in LLC-PK$_1$ cells. Chimeric cDNAs were constructed from the cDNAs for Gα$_{i-3}$ and Gα$_{i-2}$ and introduced into a pMXX eukaryotic expression vector containing a mouse metallothionein-I promotor. Stably transfected cell lines were produced that expressed either Gα$_{i-2/3}$ or Gα$_{i-3/2}$ chimeric proteins. Chimeric and endogenous Gα$_i$ proteins were detected in cells using specific carboxy-terminal peptide antibodies. Immunofluorescence staining was used to localize endogenous and chimeric Gα$_i$ proteins in LLC-PK$_1$ cells. The staining of chimeric proteins was detected as an increased intensity of staining on membranes containing endogenous Gα$_i$ proteins. Using confocal microscopy and image analysis we localized Gα$_{i-2}$ to a specific sub-domain of the lateral membrane of polarized cells, the chimeric Gα$_{i-3/2}$ protein was then shown to colocalize with endogenenous Gα$_{i-2}$ in the same lateral plasma membrane domain. The chimeric Gα$_{i-2/3}$ protein colocalized with endogenous Gα$_{i-3}$ on Golgi membranes in LLC-PK$_1$ cells. These results show that chimeric Gα$_i$ proteins were targeted to the same membrane domains as endogenous Gα$_i$ proteins and the specificity of their membrane targeting was conferred by the carboxy-terminal end of the proteins. These data provide the first evidence for specific targeting information contained in the carboxy termini of Gα$_i$ proteins, which appears to be independent of amino-terminal membrane attachment sites in these proteins.

Key words: G protein, targeting signal, plasma membrane, Golgi, immunofluorescence

INTRODUCTION

Heterotrimeric guanine nucleotide-regulatory (G) proteins are involved in the modulation of signal transduction for an increasingly diverse number of receptor-effector systems. These G proteins are composed of a GTP-binding α subunit and a βγ complex, with the specificity for receptor-effector interactions residing largely in the α subunits. The non-membrane spanning Gαβγ proteins remain associated with the cytoplasmic surface of membranes through a series of attachments that may be mediated by one or more of the subunits (Jones et al., 1990; Juhnn et al., 1992; Mumby et al., 1990; Simonds et al., 1991; Sternweiss, 1986). During the activation of G proteins and the dissociation of the α subunit from βγ, the α subunit (with the exception of transducin) remains tightly associated with membranes (Mumby et al., 1990). It has been demonstrated that post-translational modifications for these non-membrane spanning proteins, specifically fatty acyl modifications, are required to sustain membrane attachment for at least one family of α subunits, the Gαi proteins. Myristoylation of residues at the amino terminus of the αi subunits of the pertussis toxin-sensitive category of G proteins, G$_i$ and G$_o$, was found to be obligatory for membrane attachment since mutant forms lacking the myristoylation site resulted in the loss of their membrane association (Mumby et al., 1990).

The presence of Gα$_i$ proteins, once thought to reside solely on the plasma membrane, has now been described on the membranes of intracellular organelles, such as the endoplasmic reticulum (Audigier et al., 1988), the Golgi complex (Stow et al., 1991a), endosomal membranes (Ali et al., 1989) and possibly the nucleus (Crouch, 1991). Different G protein α subunits may also be segregated into specific sub-domains of plasma membranes. In rat kidney, different Gα$_s$ and Gα$_i$ proteins have been localized to either the apical or basolateral plasma membrane domains of individual epithelial cells along the nephron (Stow et al., 1991b). This geographic diversity presumably dictates the functional consequences of many receptor-G protein-effector systems in selective membranes. The targeting of these highly homologous Gα proteins, particularly the Gα$_i$ proteins, to such a variety of intracellular membranes and sub-domains of the plasma membrane thus presents a complex trafficking problem.

While the sequences required for myristoylation and membrane attachment of G protein subunits are known (Jones et al., 1990; Mumby et al., 1990), there are no data to define the specific regions of $G\alpha_i$ subunits required for targeting to distinct plasma or intracellular membranes. We have demonstrated recently that two homologous α subunits of the Gi family, $G\alpha_{i-2}$ and $G\alpha_{i-3}$, are selectively targeted to distinct and specific membranes in renal epithelial cells (Ercolani et al., 1990; Stow et al., 1991a). In LLC-PK$_1$ cells, the $G\alpha_{i-2}$ is targeted to the basolateral plasma membrane, whereas $G\alpha_{i-3}$ is targeted to Golgi membranes. The pertussis toxin-sensitive $G\alpha_{i-2}$ protein is the probable mediator of inhibition of adenylyl cyclase at the plasma membrane (Ercolani et al., 1990; Skorecki et al., 1987). In contrast, both native and overexpressed $G\alpha_{i-3}$ on Golgi membranes are involved in regulating trafficking through the secretory pathway (Stow et al., 1991a).

The present study was undertaken to define the regions of the $G\alpha_{i-2}$ and $G\alpha_{i-3}$ subunits that are necessary for specific targeting to plasma and Golgi membranes, respectively. Chimeric $G\alpha_i$ proteins were stably expressed in polarized epithelial LLC-PK$_1$ cells. These studies revealed that the sites for specific targeting to intracellular membranes and plasma membranes reside within specific segments of the carboxy termini of the $G\alpha_i$ proteins. Thus, the signals for targeting and membrane attachment in these proteins are different.

MATERIALS AND METHODS

Construction of plasmids and production of stably-transfected cell lines.

LLC-PK$_1$ cells, a polarized epithelial cell line derived from pig kidney, were grown as described previously in Dulbecco's modified Eagle's medium with 10% FCS, in 5% CO_2 (Ercolani et al., 1990). Vectors for the transfection of cDNAs of rat $G\alpha_{i-2}$ and $G\alpha_{i-3}$ were constructed as described previously (Ercolani et al., 1990; Stow et al., 1991a). Chimeric $G\alpha_{i-2/3}$ and $G\alpha_{i-3/2}$ cDNAs were constructed as described in Fig. 1 and cloned into pMXX, a vector containing a mouse metallothionein-1 inducible promoter, as we have described previously (Ercolani et al., 1990). The vectors were cotransfected with pSV2neo into LLC-PK$_1$ cells by the calcium phosphate precipitation method (Gorman et al., 1982). Cells containing stably integrated plasmids were selected by resistance to geneticin (G418) (GIBCO) at 1.5 mg/ml. To identify stable transfectants containing rodent chimeric genes, genomic DNA from geneticin resistant cells were utilized as templates in the polymerase chain reaction. Rodent-specific oligonucleotides for $G\alpha_{i-2}$ and $G\alpha_{i-3}$ flanking the BamHI ligation site for chimeric $G\alpha_{i-3/2}$ or $G\alpha_{i-2/3}$ cDNAs were utilized. Amplification products of 262 bp ensured specificity of rodent chimeric cDNAs as these primers would potentially amplify larger porcine DNA products due to interruption by introns 5 and 6 in both the porcine $G\alpha_{i-2}$ and $G\alpha_{i-3}$ genes:

Rat.$G\alpha_{i-2}$ mRNA forward primer: 5'-GGATGTGCTGCGGACC-CGT-3'

Rat.$G\alpha_{i-2}$ mRNA reverse primer: 5'-AGATGTTCTTCGGAC-GAGA-3'

Rat.$G\alpha_{i-3}$ mRNA forward primer: 5'-GGATGTGCTGCGGACC-CGT-3'

Rat.$G\alpha_{i-3}$ mRNA reverse primer: 5'-AAACCATTTGTTGTTA-CAA-3'

LLC-PK$_1$ cell lines stably transfected with pMXX $G\alpha_{i-2/3}$ were designated as LLC-PK$_{1(2/3)}$ cells and stable cells lines transfected with pMXX $G\alpha_{i-3/2}$ were designated as LLC-PK$_{1(3/2)}$ cells.

Antibodies

Polyclonal antibodies to specific decapeptides from $G\alpha_i$ subunits were obtained from Allen Spiegel, NIH or were purchased from Dupont (Wilmington, DE). The AS antibody recognizes a carboxy-terminal epitope of $G\alpha_{i-2}$ (Spiegel et al., 1990) and thus also recognizes the chimeric $G\alpha_{i-3/2}$ protein, which contains the same peptide. The EC antibody specifically recognizes the carboxy-terminal epitope of $G\alpha_{i-3}$ (Spiegel et al., 1990) and it can be used for specific immunoblotting and immunolocalization of $G\alpha_{i-3}$ in LLC-PK$_1$ cells (Stow et al., 1991a); the EC antibody also recognizes the chimeric $G\alpha_{i-2/3}$ protein. The monoclonal antibody to the α-subunit of the Na$^+$,K$^+$-ATPase (Caplan et al., 1990) was obtained from Dr Michael Caplan, Yale University and the antibody to the ZO-1 tight junction protein (Stevenson et al., 1986) was provided by Dr Dan Goodenough, Harvard Medical School. Goat anti-rabbit IgGs conjugated to FITC, rhodamine or alkaline phosphatase were obtained from Vector Laboratories (Burlingame, CA).

Immunofluorescence staining and western blotting

Immunofluorescence staining of $G\alpha_i$ proteins in cells was carried out as described previously (Ausiello et al., 1992; Stow et al., 1991a). Cells on coverslips were fixed either in 4% paraformaldehyde for 1 hour or in methanol/acetic acid (3:1 ratio). After fixation, cells were permeabilized with 0.1% Triton X-100, incubated sequentially in diluted specific $G\alpha_i$ peptide antibodies, followed by second antibodies conjugated to FITC or rhodamine. Stained cultures were mounted in PBS/glycerol with 1% n-propyl-gallate and viewed by conventional epifluorescence on a Nikon Microphot microscope or by confocal imaging on a Bio-Rad MRC 600 system.

Total microsomal membranes (100,000 **g** pellet) and cytosol fractions (100,000 **g** supernatant) were prepared, as described (Stow et
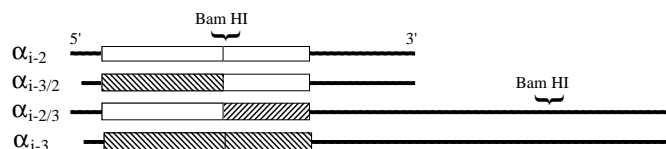

Fig. 1. $G\alpha_i$ cDNAs. Plasmids Gi-f2 and Gi-f3 encoding rodent cDNAs for $G\alpha_{i-2}$ and $G\alpha_{i-3}$ were kindly provided by R. Reed (Johns Hopkins). A BamHI restriction site was identified in the coding region of each cDNA that would allow for the in frame translation of the amino acids WIC at positions 785 and 716 in $G\alpha_{i-2}$ and $G\alpha_{i-3}$, respectively. Each rodent cDNA was derived by digesting Gi-f2 and Gi-f3 with EcoRI, followed by purification of the respective 1748 bp and 3072 bp fragments. BamHI full digestion of 1748 bp $G\alpha_{i-2}$ cDNA yielded a 785 bp fragment (encoding 151 bp of 5' untranslated region and 634 bp of amino-terminal coding region) and a 963 bp fragment (encoding 433 bp of carboxy-terminal coding region and 530 bp of 3' untranslated region). BamHI partial digestion of 3072 bp $G\alpha_{i-3}$ cDNA yielded a 716 bp fragment (encoding 85 bp of 5' untranslated region and 631 bp of amino-terminal coding region) and a 2356 bp fragment (encoding 433 bp of carboxy-terminal coding region and 1923 bp of 3' untranslated region). Untranslated regions of each cDNA are depicted as black lines whereas rectangular open boxes are $G\alpha_{i-2}$ coding region and rectangular striped boxes are $G\alpha_{i-3}$ coding region. For construction of $G\alpha_{i-3/2}$ cDNA the 716 bp $G\alpha_{i-3}$ fragment was ligated to the 963 bp $G\alpha_{i-2}$ fragment. For construction of $G\alpha_{i-2/3}$ cDNA the 785 bp $G\alpha_{i-2}$ fragment was ligated to the 2356 bp $G\alpha_{i-3}$ fragment. Each cDNA was then ligated into the EcoRI polylinker of the pMXX eukaryotic expression vector containing a mouse metallothionein I promoter-enhancer. Sense orientation for each plasmid was confirmed by KpnI digestion of pMXX ($\alpha_{i-3/2}$) and KpnI/PstI digestion for pMXX ($\alpha_{i-2/3}$) followed by dideoxy nucleic acid sequencing. Each $G\alpha_i$ cDNA is drawn to scale.

al., 1991a), from cultures of stable LLC-PK$_{1(2/3)}$ and LLC-PK$_{1(3/2)}$ cell lines to analyze chimeric proteins by western blotting with specific antibodies. Aliquots of membranes and cytosols, matched for protein content, were electrophoresed on a 12% SDS-polyacrylamide gel and then transferred electrophoretically to Immobilon-P membrane (Millipore Corporation, Bedford, MA). Western blotting was performed with peptide antibodies to the Gα$_i$ subunits followed by alkaline phosphatase-labeled secondary antibody (Stow et al., 1991a).

In all immunofluorescence and western blotting experiments, untreated LLC-PK$_{1(2/3)}$ and LLC-PK$_{1(3/2)}$ cells, expressing only native Gα$_i$ subunits, were compared to cells exposed to 5 μM CdCl$_2$ for 16 hours, to induce additional expression of the transfected, chimeric cDNAs. Chimeric proteins in both procedures were detected with the AS antibody to recognize both endogenous Gα$_{i-2}$ and chimeric Gα$_{i-3/2}$ proteins or with the EC antibody to recognize both endogenous Gα$_{i-3}$ and chimeric Gα$_{i-2/3}$ proteins.

Quantitation of fluorescent signals on membrane domains using confocal microscopy

In order to quantitate the intensity of the fluorescent signal generated by immunofluorescence labelling of Gα$_i$ proteins, multiple, random images of stained cells were collected on the laser confocal microscope as described previously (Ausiello et al., 1992; Stow et al., 1991a). Histograms were generated for each image to quantitate pixel intensity in a designated perinuclear area (for Golgi staining) or along a line intersecting cell borders (for plasma membrane staining) using the Bio-Rad MRC 600, CM software (Bio-Rad Microsciences, Cambridge, MA). To make direct comparisons between plasma membrane staining or Golgi staining in induced versus uninduced cells, the pixel intensities of 15-20 images for each condition were measured and used to calculate a mean fluorescence intensity. Some images were also pseudocoloured to highlight the distribution of staining at two different intensity ranges within cells.

RESULTS

Localization of native Gα$_{i-2}$

In order to examine the targeting of chimeric Gα$_i$ proteins to membrane domains it was necessary to know the precise distribution of native and overexpressed Gα$_{i-2}$ and Gα$_{i-3}$ in LLC-PK$_1$ cells. In previous studies we have established, by immunocytochemistry at the light and electron microscopy levels, that native and overexpressed Gα$_{i-3}$ are localized on the cytoplasmic face of Golgi cisternae membranes (Stow et al., 1991a). We have previously localized both the native and overexpressed Gα$_{i-2}$ on basolateral plasma membranes of LLC-PK$_1$ cells using epifluorescence microscopy on cell monolayers (Ercolani et al., 1990). However, using this technique it was not possible to precisely define the localization of Gα$_{i-2}$ within sub-domains of the basolateral plasma membrane. Now, using confocal microscopy, we have been able to more accurately determine the localization of Gα$_{i-2}$ in a sub-domain on the lateral plasma membrane of polarized LLC-PK$_1$ cells. LLC-PK$_1$ cells grown as confluent monolayers on filters were stained by immunofluorescence with the AS antibody and then examined on a confocal scanning laser microsocpe. Optical sections were produced at 1 μm intervals through the monolayer to construct a Z-series of images showing staining at each level of cells from the apex to the base. Analysis of the images (Fig. 2) showed that Gα$_{i-2}$ is not distributed randomly over the basolateral membrane; staining was generally absent from the basal membrane and was confined to the lateral cell membrane. The localization of Gα$_{i-2}$ was compared to that of two other membrane proteins. The ZO-1 protein is localized in

tight junctions and the α-subunit of Na$^+$,K$^+$-ATPase is a basolateral membrane protein on polarized epithelial cells. The images shown in Fig. 2 represent a Z-series of optical sections taken at 1 μm intervals through three separate LLC-PK$_1$ mono-
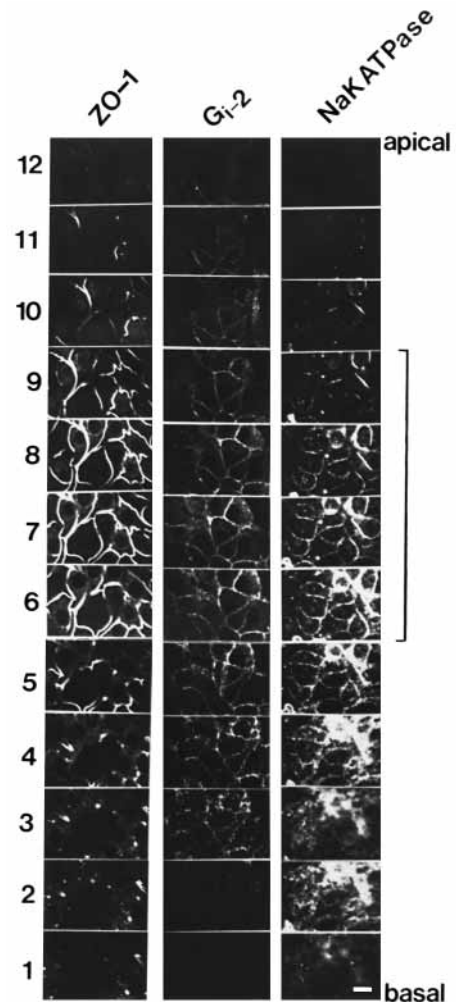
Fig. 2. Localization of Gα$_{i-2}$ by immunofluorescence staining and confocal imaging. Confluent monolayers of LLC-PK$_1$ cells grown on filters, were stained with specific antibodies and examined by confocal laser microscopy. A set of images at 1 μm intervals was created to form a Z-series through the cells from the basal membrane (image 1) through to the apex of the cells (image 12) for cultures stained with each antibody. The staining in each image represents membrane staining around the circumference of each cell. The ZO-1 tight junction protein (first strip) is localized in a band around the lateral membranes (images 6-9) of the cells. There is intense staining of the lateral membrane outlining each cell, there is no staining of the apical or basal membranes, nor any intracellular staining. The staining of Gα$_{i-2}$ (center strip) is also most intense on the lateral membranes (images 6-9), with no staining on the apical membrane and weaker staining in more basal sections. The Gα$_{i-2}$ antibody gives a weak cytoplasmic staining, as also seen by epifluorescence microscopy (see Fig. 4). There is Na$^+$,K$^+$-ATPase staining (last strip) on the basal membranes (images 2-4) of these cells, which appears as more diffuse staining across the sections, as well as staining all along the lateral membranes. The bracket indicates the portion of the lateral membrane, beginning at the level of the tight junction (images 9-6), in which there is staining of Gα$_{i-2}$, overlapping with staining of ZO-1 and Na$^+$,K$^+$-ATPase.

layers that were stained with antibodies to the membrane proteins ZO-1, Na$^+$,K$^+$-ATPase, and Gα_{i-2}, respectively. ZO-1 was found, as expected, in a relatively compact area of the lateral membrane at the level of the tight junctions and was absent from the apical and basal membranes. Na$^+$,K$^+$-ATPase α subunit was distributed over most of the basal and the lateral membranes, with the greatest concentration on the basal surface of these LLC-PK$_1$ cells. Gα_{i-2} staining was absent from the apical membrane and from the basal membrane but was present on the lateral cell membranes. Staining of the Gα_{i-2} subunit was most heavily concentrated in a 4 μm band on the lateral membrane, in a location that largely overlaps with the ZO-1 staining at the tight junction. The concentration of Gα_{i-2} in this lateral membrane band was most clearly demonstrated when images were projected in the XZ plane (Fig. 3). These data confirm our previous observation that Gα_{i-2} is a basolateral membrane protein in LLC-PK$_1$ cells (Ercolani et al., 1990). However, we can now see that the Gα_{i-2} subunit is not distributed randomly over the basolateral membrane but is targeted to a specific sub-domain, near the tight junction on the lateral membrane of these cells.

The analysis of staining by confocal Z-series images was also used for accurate localization of chimeric Gα_i proteins on plasma membranes. However, since confocal Z-series imaging was not informative for analysis of Golgi staining in these cells, the staining of Gα_{i-3} or chimeric Gα_i proteins on the Golgi membranes was performed by direct epifluorescence microscopy.

Expression of chimeric proteins

Stable clones of LLC-PK$_{1(2/3)}$ and LLC-PK$_{1(3/2)}$ cells were tested for expression of chimeric Gα_i proteins by western blotting of microsomal membranes. The proteins encoded by endogenous and chimeric cDNAs were expected to be of
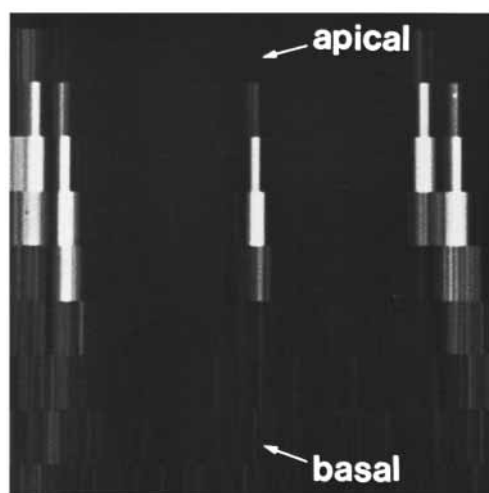
similar size, thus Gα_{i-2} and G$\alpha_{i-2/3}$ were 40 kDa while Gα_{i-3} and G$\alpha_{i-3/2}$ were 41 kDa, respectively. Indeed, protein bands corresponding to native and chimeric Gα_is were not resolved from each other and were superimposed on SDS-polyacrylamide gels. Expression of the chimeric proteins was seen as increased intensity of the stained, overlapping protein bands in membranes from CdCl$_2$-induced cells compared to uninduced cells (Fig. 4), as shown previously for the induced expression of native Gα_{i-2} and Gα_{i-3} in LLC-PK$_1$ cells (Ercolani et al., 1990).

Membrane fractions from both the LLC-PK$_{1(2/3)}$ and LLC-PK$_{1(3/2)}$ cell lines showed a 2-3 fold increase in gel band intensity following induction of expression of the chimeric proteins, consistent with the presence of newly expressed chimeric proteins in the induced cultures (Fig. 4). This also verifies that induction of the chimeric cDNA results in expression of the appropriate chimeric protein. Neither the chimeric proteins nor the native proteins were found in cytosol fractions, indicating that the chimeric Gα_i proteins remain completely associated with membranes.

Localization of chimeric Gαi proteins

The specific carboxy-terminal peptide antibodies were used to localize chimeric Gα_i proteins by immunofluorescence staining in transfected LLC-PK$_1$ cells. Overexpression of the chimeric proteins was only detected as a change in the intensity or position of staining in CdCl$_2$-induced cells compared to staining of native proteins alone in uninduced cells. The localization of chimeric proteins was analyzed by regular epifluorescence microscopy of monolayers on coverslips (see Figs 5 and 8) and quantitated by confocal laser imaging (see Figs 6, 7 and 9).

In uninduced LLC-PK$_{1(3/2)}$ cells, staining with the AS antibody detected only the native Gα_{i-2}, which was found predominantly on the basolateral plasma membrane (Fig. 5a). The immunofluorescence staining of Gα_{i-2} in whole monolayers, viewed by epifluorescence microscopy appeared as intense

Fig. 3. Confocal imaging of Gα_{i-2} staining in an XZ projection. A series of images of LLC-PK$_1$ cells stained with the AS antibody were collected. Single images were projected as an XZ image constructed of 14 steps (1 μm) with 15 scans/step from the apical to the basal membrane of the cells. There is weak staining along lateral and basal aspects of three adjacent cells. There is intense staining in an area of the lateral membranes of each cell corresponding to the location of Gα_{i-2}.

Fig. 4. Western blot analysis of native and chimeric Gαi proteins. Microsomal membranes were prepared from LLC-PK$_1$ cells, electrophoresed on SDS-PAGE gels and transferred for immunoblotting. Membranes from the LLC-PK$_{1\ (2/3)}$ cells were probed with the EC antibody to label native Gα_{i-3} (41 kDa) and the G$\alpha_{i-2/3}$ chimeric protein. Membranes from LLC-PK$_{1\ (3/2)}$ cells were reacted with the AS antibody, which recognizes the native Gα_{i-2} (40 kDa) and the G$\alpha_{i-3/2}$ protein. In both cell lines the amount of immunoreactive protein was compared in membranes from uninduced cells (−) and in membranes from cells treated with 5 μM CdCl$_2$ (+) to induce expression of the chimeric proteins. In both cases there was a more intense staining of the 40 kDa and 41 kDa bands in induced cells, which is consistent with the appearance of the chimeric proteins superimposed over the native Gα protein bands. This also shows that the chimeric proteins have been correctly processed and are migrating at the expected molecular masses.

Fig. 5. Immunofluorescence localization of chimeric Gα$_{i-3/2}$ protein. Untreated LLC-PK$_{1 (3/2)}$ cells (−Cd) and cells treated with 5 μM CdCl$_2$ (+Cd) were fixed and stained w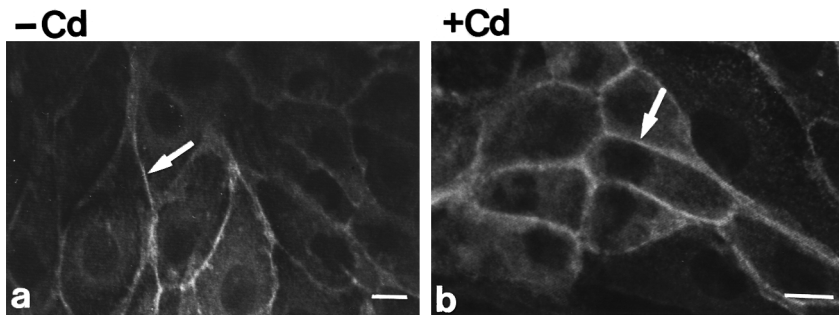ith the AS antibody. (a) In uninduced cells, there is staining of native Gα$_{i-2}$ on the basolateral membranes (arrow), which appears as a line around the border of the cells, and faint diffuse cytoplasmic staining. (b) Following induction and expression of the chimeric proteins, there is staining of both the native Gα$_{i-2}$ and chimeric Gα$_{i-3/2}$ proteins on the basolateral membranes. The membrane staining is now more intense due to the superimposition of the two Gα proteins (arrow). Bars, 10 μm.

staining of the cell borders in a cobblestone-like pattern that is typical of plasma membrane staining. In induced LLC-PK$_{1(3/2)}$ cells, now expressing both the endogenous Gα$_{i-2}$ protein and the Gα$_{i-3/2}$ chimeric protein, there was also staining of the basolateral cell membranes and this staining was notably more intense than staining in comparable monolayers of uninduced cells (Fig. 5b). The staining of the endogenous and chimeric proteins appeared to be completely superimposed, there was no de novo staining of Golgi membranes or any other membranes in these cells following induction of the expression of the chimeric protein. There was some cytoplasmic staining with the AS antibody, as we have previously reported, and the staining was the same in both induced and uninduced cells.

Monolayers of induced LLC-PK$_{1(3/2)}$ cells, stained with the AS antibody, were also examined by confocal microscopy, to compare the staining of the Gα$_{i-3/2}$ chimeric protein, with that of the endogenous Gα$_{i-2}$ protein in sub-domains of the plasma membrane (Fig. 6). Confocal images, analyzed as a Z-series, confirmed that in induced LLC-PK$_{1(3/2)}$ cells the staining of both the endogenous Gα$_{i-2}$ protein and the newly expressed Gα$_{i-3/2}$ protein was completely superimposed (Fig. 6); the most intense staining was found in a 4 μm sub-domain of the lateral plasma membrane, which corresponded exactly to the most concentrated staining of Gα$_{i-2}$ in normal cells (as seen in Figs 2 and 3).

The immunofluorescent staining of plasma membranes in induced and uninduced LLC-PK$_{1(3/2)}$ cultures was quantitated by image analysis on the confocal microscope. The average maximum pixel intensity in uninduced cells and induced cells was 101 and 189, respectively (Fig. 7), confirming that there was indeed more intense staining of plasma membranes following expression of the chimeric protein, and consistent with the additive presence of the chimeric protein detected in these cells by western blotting. Also, following induction in the LLC-PK$_{1(3/2)}$ cells, there was no increase in Golgi staining with the EC antibody and no staining with this antibody of the plasma membrane (Fig. 7 and see also Fig. 5b). The EC antibody recognizes only the carboxy terminus of native Gα$_{i-3}$ and does not recognize the chimeric Gα$_{i-3/2}$ protein. Treatment of mock-transfected, or untransfected LLC-PK$_1$ cells with CdCl$_2$ did not result in any alteration or increase in the intensity of staining with AS or EC peptide antibodies (data not shown). These data show that: (i) the endogenous Gα$_{i-2}$ protein and the chimeric Gα$_{i-3/2}$ protein, which share the same carboxy-terminal sequence, are both recognized in intact cells

by the AS antibody; (ii) staining of both proteins is superimposed on the plasma membrane resulting in an increase in fluorescence intensity of plasma membrane staining; (iii) there was no staining of Gα$_{i-3/2}$ protein detected on any other membranes in the cell; and (iv) the native Gα$_{i-2}$ and Gα$_{i-3/2}$ proteins are colocalized on the same sub-domain of the lateral plasma membrane of LLC-PK$_1$ cells.

The localization of the converse chimeric protein, Gα$_{i-2/3}$, was carried out by immunofluorescence staining in LLC-PK$_{1(2/3)}$ cells with the EC antibody, which recognizes the Gα$_{i-3}$ carboxy terminus. This demonstrated that the overexpressed chimeric Gα$_{i-2/3}$ protein is targeted, along with native Gα$_{i-3}$, to Golgi membranes (Fig. 8). In uninduced LLC-PK$_{1(2/3)}$ cells, where there is only endogenous Gα$_{i-3}$, there was staining of the perinuclear Golgi complex, and faint staining of the cytoplasm (Fig. 8a) as we have demonstrated previously (Stow et al., 1991a). Following CdCl$_2$ induction, the Gα$_{i-2/3}$ chimeric protein was expressed and its staining was superimposed over the endogenous Gα$_{i-3}$ protein on the perinuclear Golgi complex (Fig. 8b). There was an increase in the intensity of
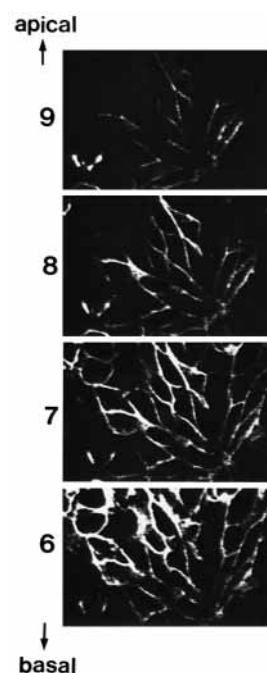
Fig. 6. Confocal localization of chimeric Gα$_{i-3/2}$ on lateral cell membranes. LLC-PK$_{1 (3/2)}$ cells were treated with 5 μM CdCl$_2$ to induce expression of the Gα$_{i-3/2}$ chimera. The cells were fixed, stained with the AS antibody and examined by confocal imaging. A Z-series of images was produced, as described above and the panels corresponding to images 6-9 in Fig. 2, are shown here. The lateral membrane staining of Gα$_{i-3/2}$ and Gα$_{i-2}$ is superimposed and is localized in the same images as native Gα$_{i-2}$ alone (see Fig. 2), showing that Gα$_{i-3/2}$ is targetted to the same sub-domain of the lateral membrane as the native subunit.
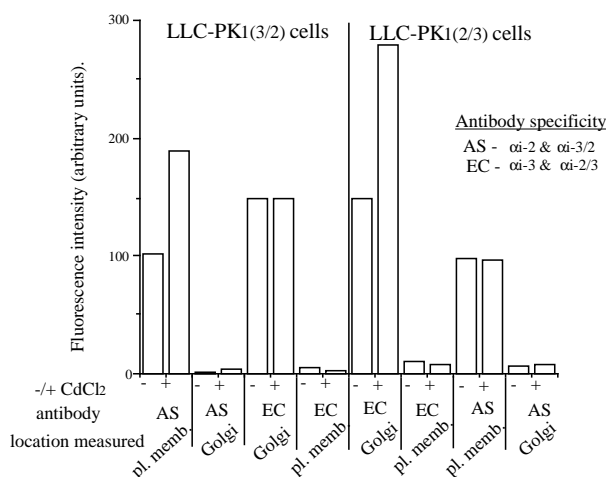
Fig. 7. Quantitation of fluorescence intensities. LLC-PK$_{1 (2/3)}$ and LLC-PK$_{1 (3/2)}$ cells were treated with CdCl$_2$ overnight to induce expression of the Gα$_{i-2/3}$ and Gα$_{i-3/2}$ chimeric proteins, respectively. Untreated cells, expressing only native Gα$_i$ subunits, were used for comparison. Cells were stained with the AS and EC antibodies, images were collected on the confocal microscope and image analysis, using the MRC 600 software as described in the text, was performed to quantitate plasma membrane and Golgi staining. The graph shows average maximum pixel intensities recorded from 15-25 measurements for each antibody and each site. The AS antibody stained the Gα$_{i-2}$ and Gα$_{i-3/2}$ proteins on plasma membranes but did not give any Golgi staining, conversely the EC antibody recognized both Gα$_{i-3}$ and Gα$_{i-2/3}$ on the Golgi but did not stain plasma membranes.

the perinuclear Golgi staining following expression of the chimeric protein. Quantitation of images by confocal microscopy revealed that the average fluorescence intensity increased from 151 to 280, respectively, following induction of chimeric protein expression (Fig. 7). There was no evidence
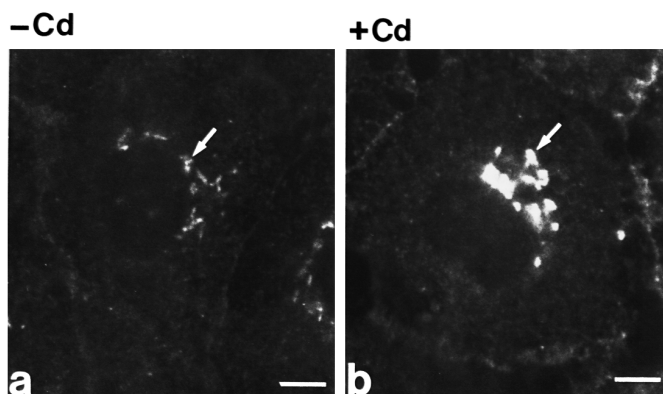

Fig. 8. Immunofluorescence staining of chimeric Gα$_{i-2/3}$ protein. (a) Untreated LLC-PK$_{1 (2/3)}$ cells (−Cd), stained with the EC antibody, show staining of native Gα$_{i-3}$ on the Golgi complex, which appears as a perinuclear patch of membranes (arrow). (b) Following induction with 5 μM CdCl$_2$ (+Cd) there is expression of the Gα$_{i-2/3}$ chimera, which is also localized on Golgi membranes superimposed on the endogenous subunit and there is now more intense staining of the perinuclear Golgi complex (arrow). Bars, 10 μm.

that the chimeric protein, labeled with the EC antibody, was localized on plasma membranes or any membranes other than Golgi membranes (Fig. 7 and see Fig. 9). As expected, there was no increased staining in LLC-PK$_{1(2/3)}$ cells with the AS antibody, which recognizes the carboxy terminus of native Gα$_{i-2}$ (Fig. 6). The confocal imaging of EC staining in LLC-PK$_{1(2/3)}$ cells is shown in the pseudocoloured images in Fig. 9. The images are coloured with yellow to show less intense staining and red to show the most intense staining obtained with the EC antibody. There is staining of native Gα$_{i-3}$ in perinuclear Golgi regions (Fig 9A), and the staining of Gα$_{i-3}$ and Gα$_{i-2/3}$ are superimposed (Fig 9B) resulting in an increase in fluorescent intensity and a change from yellow to red in most of these Golgi regions. These images show very clearly that there is no staining of Gα$_{i-2/3}$ on the plasma membrane or other membranes in these cells.

These findings reflect all of the same parameters found for the expression and localization of the Gα$_{i-3/2}$ chimera described above. The major difference being that, while Gα$_{i-3/2}$ was targeted to the plasma membrane, we now find that the Gα$_{i-2/3}$ chimeric protein is targeted to the Golgi membranes, in the same manner as the native Gα$_{i-3}$.

DISCUSSION

We have demonstrated previously that, despite the high homology between the rat Gα$_{i-2}$ and Gα$_{i-3}$ subunits, there is selective targeting of these α$_i$ subunits to distinct membranes in developing and polarized renal epithelial cells (Ercolani et al., 1990; Holtzman et al., 1991; Stow et al., 1991a). In the polarized LLC-PK$_1$ cells, Gα$_{i-3}$ is on Golgi membranes and Gα$_{i-2}$ is on the basolateral plasma membrane. In previous studies, using epifluorescence microscopy, it was not possible to determine whether the Gα$_{i-2}$ was distributed over the entire basolateral plasma membrane, or whether it had a more restricted distribution. Now, by using confocal laser microscopy, we show that Gα$_{i-2}$ is indeed concentrated within a restricted sub-domain of the lateral membrane of these cells and that the targeting of this subunit is a highly selective process. The functional consequences of having Gα$_{i-2}$ localized to this particular domain of the plasma membrane are not known. However, because this Gα$_{i-2}$ is believed to be involved in regulation of adenylyl cylcase (Ercolani et al., 1990; Skorecki et al., 1987), it suggests that the adenylyl cyclase and associated receptor(s) may also be localized in this domain. It is not currently known how Gα$_{i-2}$, or other G proteins, are retained within a restricted domain of a membrane. The interactions of Gα$_{i-2}$ with its βγ subunits and with other membrane proteins may well be important for initially capturing and then maintaining Gα$_{i-2}$ at its membrane attachment site.

Having established that the endogenous and overexpressed Gα$_{i-2}$ and Gα$_{i-3}$ proteins in LLC-PK$_1$ cells are targeted to such well-defined and distinct membrane domains, this then provided a system in which to study the targeting of chimeric proteins. Other functional domains of Gα$_i$ subunits have been studied using chimeric proteins. Chimeras made between Gα$_s$ and Gα$_{i-2}$ were found to be functional and able to activate adenylyl cyclase (Osawa et al., 1990; Woon et al., 1989). Some studies have also investigated the intracellular distribution of

Fig. 9. Pseudocoloured images of staining in LLC-PK$_1$ $_{(2/3)}$ cells. Untreated cells, expressing only native Gα$_{i-3}$ (A), or cells induced with CdCl$_2$, to express both Gα$_{i-3}$ and Gα$_{i-2/3}$ proteins (B) were stained with the EC antibody and confocal images were collected. Images were pseudocoloured to show areas of specific immunofluorescence staining at two intensity ranges with yellow (representing pixel intensities 85-120) and red (representing pixel intensities 120-255). Most of the perinuclear Golgi staining (arrow) of Gα$_{i-3}$ shows as yellow with only small patches of the most intense staining (red) (A). Co-expression of Gα$_{i-2/3}$ results in only staining over the Golgi area again (B). These images clearly show that the chimeric protein is superimposed over the staining of native Gα$_{i-3}$ around the scattered, perinuclear Golgi complexes. As a result the pixel intensity of this staining is increased and now shows in the red range.

mutant or chimeric Gα proteins with respect to their distribution in total membranes or cytosol fractions (Juhnn et al., 1992; Mumby et al., 1990). To date however, these studies have not provided any information about the specificity of membrane-domain targeting of chimeric Gα proteins.

High levels of overexpression of Gα$_i$ subunits in LLC-PK$_1$ cells can result in mis-targeting of the Gα$_i$ subunits, but at the low levels of expression achieved with the MT-1 promoter, the chimeric proteins were found to be consistently targeted to the same membranes and were all appropriately membrane-associated, since no chimeric proteins were recovered in cytosol fractions from transfected cells. The results of immunofluorescence staining experiments show that the chimeric proteins bearing the carboxy terminus of Gα$_{i-2}$ are targeted to the plasma membrane, conversely chimeric proteins bearing the carboxy terminus of Gα$_{i-3}$ are targeted to the Golgi membrane. In both cases targeting of the chimeric proteins was faithful to the sites of the endogenous Gα$_i$ proteins, since staining of the chimeric proteins was superimposed over the staining of

endogenous Gα$_i$ proteins on these membranes and there was no staining of other membranes throughout the cells. Analysis by confocal microscopy showed that the Gα$_{i-3/2}$ chimeric protein was localized in exactly the same domain of the lateral plasma membrane as the endogenous Gα$_{i-2}$ protein.

These data now demonstrate that the sequence encoding 40% of the protein, at the carboxy-terminal end of the Gα$_{i-2}$ and Gα$_{i-3}$ subunits, is responsible for targeting to the lateral plasma membrane and the Golgi membranes, respectively. The membrane targeting signal encoded by this region appears to be independent of general membrane attachment signals for myristolyation, shown previously to be at a glycine residue in the amino terminus of these Gα$_i$ proteins (Jones et al., 1990; Mumby et al., 1990). The chimeric proteins were fully competent to attach to membranes. This suggests that the amino-terminal myristoylation signals are multipotent and are able to attach equally well to either plasma membranes or Golgi membranes. From these studies we cannot ascertain whether the chimeric Gα$_i$ proteins retained their ability to

Fig. 10. The amino acid sequences of Gα$_{i-2}$ and Gα$_{i-3}$ from the *Bam*HI site to the carboxy terminus are shown. Differences between the sequences are highlighted with conservative substitutions shown in grey and non-conservative substitutions shown in black. The region labelled Insert-3 is a region that is non-homologous to p21-RAS and is predicted to be on the surface of the protein away from its βγ association region.

interact with appropriate βγ subunits or other proteins in their respective membranes. Previous studies in different systems have concluded that the amino terminus of Gα proteins is either essential for, or at least involved in, βγ subunit interactions (Neer et al., 1988; Graf et al., 1992). However, βγ complexes have been shown to interact with different Gα subunits, suggesting that heterotrimer formation may be fairly promiscuous (Gilman, 1987). Thus, if different βγ subunits reside on Golgi and plasma membranes, it is quite likely that the endogenous and chimeric Gα$_i$ protein will form the heterotrimeric αβγ complex equally well. Further studies on the ability of the chimeric Gα$_i$ subunits to be ADP-ribosylated by pertussis toxin, which requires heterotrimer formation, and functional studies, will be required to relate the membrane domain specificity of the endogenous Gα$_i$ proteins to the chimeras.

Signals for targeting and membrane attachment of cytoplasmic, membrane-associated proteins are only beginning to be understood. The nature of the targeting signal(s) in Gα$_i$ proteins is not known. In the related, monomeric families of G proteins, some candidate targeting signals have been identified. Such signals for targeting and membrane atttachment are also often found in the carboxy terminus of these proteins. In mammalian ras proteins, a carboxy-terminal CAAX box in combination with either a polybasic region or a palmitoylation site, is required for both attachment and targeting of the proteins to the plasma membrane (Hancock et al., 1991). In the rab G protein family, a carboxy-terminal cysteine motif is essential for membrane attachment but an additional hypervariable domain, also in the carboxy terminus, was found to be necessary for targeting different rabs to selective membranes (Chavrier et al., 1991). Different members of the rho G protein family are targetted to the plasma membrane and to membranes in the endocytic pathway by specific carboxy-terminal sequences and by interactions with other proteins mediated through the rho effector domains (Adamson et al., 1992). Thus several factors may specify the membrane attachment and targeting of these monomeric G proteins, which unlike the Gα$_i$ proteins, exist in the cytosol as well as attached to membranes.

Of particular interest in the present study, is the high degree of homology between the carboxy termini of rat Gα$_{i-2}$ and Gα$_{i-3}$, there being only 14 differences in amino acid identity and only 7 amino acids reflecting non-conserved substitutions in this region (Fig. 10). It is therefore reasonable to speculate that differences in membrane targeting are encoded by these specific amino acids, between the *Bam*HI site (amino acids 213 and 214 in Gα$_{i-3}$ and Gα$_{i-2}$, respectively) and the carboxy terminus, which produce differences in the structures of Gα$_i$ proteins. Berlot and Bourne (1992) have modeled the structure of the α subunit of G$_s$ onto the coordinates derived from the structure of the GTP-bound form of p21^{ras}. In this analysis, several regions of G$_s$ have no similar regions in the p21^{ras} structure and were termed insert regions. Insert region 3 is predicted to be on a solvent-accessible surface of the Gα subunit. If one applies this modeling to the carboxy-terminal regions of Gα$_{i-2}$ and Gα$_{i-3}$ shown in Fig. 10, then 5 of the 7 non-conserved amino acid substitutions between these subunits lie within insert region 3, on an exposed face of the protein. One can further speculate that either single amino acids in this region, or unique structures resulting from the density of sequence differences in this region, might be likely candidates

for targeting signals. The presence of targeting signals in this region of the Gα$_i$ sequence would be consistent with the membrane targeting phenotypes of the chimeric proteins used in the present study.

It also remains to be shown whether or not selective membrane targeting is the result of a direct interaction of Gα$_i$ with the respective membranes, additional proteins, or through interaction with βγ subunits. However, since Gα$_i$ proteins are presumably made on free ribosomes and must move through the cytoplasm to their respective intracellular and plasma membrane domains, it seems probable that the specificity of targeting resides within the Gα$_i$ subunit itself and involves interactions with other proteins, in addition to the βγ complex. Site-specific mutagenesis will now be required, as well as knowledge of receptor and effector systems in plasma membranes and intracellular membranes, to define the targeting process.

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