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High level expression of transfected G protein α_{i3} subunit is required for plasma membrane targeting and adenylyl cyclase inhibition in NIH 3T3 fibroblasts

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The α subunits of pertussis toxin-sensitive G proteins G_{i1}, G_{i2} and G_{i3} have been shown to inhibit adenylyl cyclase in transfected cells. However, G_{i3} has recently been associated with protein transport and localized to the Golgi apparatus in a number of cell lines, rather than to the plasma membrane. We studied NIH 3T3 clones stably expressing different levels of a constitutively activated mutant of the α subunit of G_{i3} (α_{i3}-Q204L). Transfected α_{i3} subunits were localized to the Golgi apparatus in all NIH 3T3 clones. In clones expressing α_{i3}-Q204L at high levels, α_{i3} subunits were also localized to the plasma membrane. Those clones which demonstrated expression of α_{i3} at the plasma membrane showed a 40% to 60% inhibition of forskolin-induced cAMP accumulation. Transfected NIH 3T3 clones in which plasma membrane α_{i3} was undetectable, did not show inhibition of forskolin-induced cAMP accumulation. These data suggest that, unless high expression is achieved in transfected cells, α_{i3} is targeted predominantly to the Golgi, not to the plasma membrane, and does not control adenylyl cyclase activity in NIH 3T3 fibroblasts.

Fibroblasts; $G\alpha_{i3}$ (G proteins); Adenylyl cyclase; Golgi

1. INTRODUCTION

G proteins are membrane-bound heterotrimeric proteins responsible for the coupling of specific, seven transmembrane-domain receptors to their effectors [1–5]. G proteins consist of 3 subunits, α, β and γ. α subunits apparently determine the specificity of receptor–effector coupling. The G protein initially described as responsible for adenylyl cyclase inhibition, G_i, is actually a group of three distinct proteins: G_{i1}, G_{i2} and G_{i3} [1–3,6]. The α subunits of G_{i1}, G_{i2} and G_{i3} (α_{i1}, α_{i2} and α_{i3}) are closely related proteins encoded by 3 different genes [7]. At the amino acid level, α_{i1} is 86% identical to α_{i2} and 93% identical to α_{i3} [8]. The high sequence similarity suggests similar functions for all three α_i subunits. In stable transfections in fibroblasts (S. Hermouet, P. de Mazancourt and A.M. Spiegel, unpublished observations) [9–13], all three α_i proteins are able to inhibit adenylyl cyclase. However, α_i subunits differ in both function and subcellular distribution. Until recently, it was generally assumed that G proteins were targeted primarily, if not exclusively, to the plasma membrane. Yet, subpopulations of α_i subunits can be found in the cytosol and in association with subcellular organelles or cytoskeletal elements [14–19]. Particularly relevant is the observation that the α subunit of G_{i3} has been localized to both the Golgi and apical plasma membrane border in renal epithelial cells but is almost exclusively restricted to the Golgi apparatus in LLC-Pk1 cells [20,21]. In the latter case, 3-fold expression of wild-type α_{i3} slowed the rate of constitutively secreted proteoglycan through the Golgi apparatus, an observation that is consistent with a role for G_{i3} as a regulator of Golgi trafficking. A mechanism for such activity is unknown, although recent reports have implicated a heterotrimeric G protein in the regulation of coat proteins associated with vesicular transport [21–24]. We found that rat and murine fibroblasts show an apparently exclusive Golgi localization for α_{i3} (B.S. Wilson and M. Gist Farquhar, unpublished observations). How then can the inhibition of adenylyl cyclase by α_{i3} in transfected murine fibroblasts be explained? To address this question, NIH 3T3 cells were stably transfected with a constitutively activated mutant of α_{i3} (α_{i3}-Q204L), and the localization of α_{i3} and the effect on intracellular cAMP were measured in selected clones expressing various levels of α_{i3}-Q204L.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Cell culture reagents and geneticin (G418 sulfate) were purchased from Biofluids (Rockville, MD) and GIBCO/BRL. NIH 3T3 cells

were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum [25]. Medium was changed twice a week.

2.2. *Transfection of NIH 3T3 cells*

Rat cDNA encoding the α subunit of G_{i3} (kindly provided by Dr. R. Reed, Johns Hopkins University) was subjected to site-directed mutagenesis using the Amersham mutagenesis kit [26]. The mutant (Q204L) cDNA was subcloned into the modified retroviral expression vector pZipNeoSV(X) as described [27]. NIH 3T3 cells were transfected with 1 μg of DNA per 10 cm plate by calcium phosphate precipitation (Stratagene Mammalian Transfection kit) [28]. Twenty-four hours after transfection, cells were grown in selective medium containing geneticin (G 418, 0.75 mg/ml) for at least two weeks. Several geneticin-resistant cell pools were obtained. Cells from one cell pool were cloned by serial dilution. Thirty clones were selected and screened for their level of α_{i3} expression. Two NIH 3T3 clones transfected with vector alone, and six clones expressing different levels of α_{i3}-Q204L are described in this work.

2.3. *Immunoblot analysis*

Confluent cultures of NIH 3T3 clones were scraped and pelleted and washed three times in 10 ml of PBS, pH 7.5. The cell pellets were frozen (−70°C) and thawed once and homogenized in a Dounce homogenizer in 10 mM Tris buffer, pH 7.4, containing 0.25 M sucrose, 2 mM $MgCl_2$ and leupeptin (0.10 mg/ml), and centrifuged for 10 min at 1,000 rpm, 4°C. The supernatant was centrifuged for 30 min at 14,000 rpm in a microcentrifuge at 4°C. Pellets (membranes) were resuspended and stored at −70°C in 10 mM Tris buffer pH 7.4, 0.25 M sucrose and 0.10 mg/ml leupeptin. The protein concentration was determined by the Bradford method [29] with IgG (Bio-Rad) used as a standard. 50 μg of membrane proteins were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and immunoblotted with the affinity-purified anti-serum EC, specific for α_{i3} [30]. Detection of the antibody-antigen complex was by ^{125}I-labeled protein A. Autoradiograms were scanned by PhosphorImager (Molecular Dynamics).

2.4. *Immunocytochemistry studies*

Cells were grown on glass coverslips and fixed for 30 min with 2% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100. Immunolocalization of α_{i3} was accomplished using the EC antibody followed by a donkey anti-rabbit fluorescein conjugate (Jackson). Photographic exposures for all photographs were identical and print processing steps were kept uniform so that comparisons would accurately reflect the different levels of expression of α_{i3} among the clones. Selected clones were also grown to 90% confluency in 60-mm dishes, fixed with 2% formaldehyde, 10 mM periodate and 75 mM lysine for 10–15 min. Cells were then harvested in the same fixative and pelleted by centrifugation. Pellets were washed with PBS, infiltrated with polyvinylpyrrolidone (PVP) and then frozen. Semithin sections (0.5–1.0 μm) cut from frozen specimens were used for immunofluorescence studies with the EC antibody. Micrographs were taken using an Axiophot (Zeiss, Burbank, CA) equipped for fluorescence microscopy.

2.5. *Cyclic AMP assays*

Cells were plated in triplicate at 50,000 cells in 1 ml per well and grown to confluency in DMEM with 10% calf serum in 24-well plates (previously coated with fibronectin, 1 μg/ml in 1 ml PBS, for 60 min at 37°C). The cells were washed with DMEM, then incubated at 37°C in DMEM (1 ml per well) containing 10 mM Na HEPES pH 7.5, 1 mM IBMX with or without forskolin 50 μM. After 20 min, the incubation medium was removed and the cells were lysed (30 min, 4°C) with 0.5 ml of 0.1 M HCl/0.1 mM $CaCl_2$. The plates were stored at −20°C. After thawing, samples were acetylated by adding 10 μl of acetic anhydride/triethylamine (1:2.5, vol/vol). cAMP was measured using a radioimmunoassay [31] and protein content of each well was determined to normalize for differences in cell number. Results presented here are mean values ± SD of 3 different experiments in triplicate. For statistical analysis, we used Student–Newman–Keuls' test.

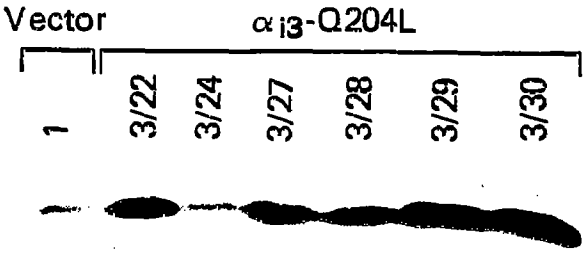

Fig. 1. Level of expression of α_{i3} in transfected NIH 3T3 clones. Crude membrane proteins (50 μg) were resolved by SDS/10% PAGE and transferred to nitrocellulose. The blots were incubated with EC antiserum. Detection of the antibody-antigen complex was by ^{125}I-labeled protein A.

3. RESULTS

3.1. *Expression of α_{i3}-Q204L in NIH 3T3 clones*

NIH 3T3 cells express α_{i2} (40 kDa) and α_{i3} (41 kDa), but not α_{i1} (41 kDa). Expression of α_{i3} is measured with antiserum EC, specific for α_{i3} [30]. The level of expression of transfected α_{i3}-Q204L in crude membrane preparations from cell pools was good, showing on average a 5-fold increase (S. Hermouet, P. de Mazancourt and A.M. Spiegel, unpublished observations). Six NIH 3T3 clones expressing α_{i3}-Q204L were selected: the level of expression of α_{i3} in these cells varies from low (clone 3/24, less than 2-fold increase) to intermediate (clones 3/22, 3/27, 3/28, 3- to 5-fold increase) or high (clones 3/29, 3/30, 10-fold increase) (Fig. 1).

3.2. *Cyclic AMP accumulation*

We measured intracellular cAMP levels in intact cells, with or without incubation with 50 μM forskolin (Fig. 2). Two NIH 3T3 clones transfected with vector alone (also referred to as control cells) and the six NIH 3T3 clones transfected with α_{i3}-Q204L cDNA described in Fig. 1 were studied. The forskolin-induced cAMP accumulation was significantly decreased in clones 3/28 (15.5 ± 1.2 nmol/mg/20 min; 62% of control), 3/29 (11.2 ± 2.1 nmol/mg/20 min; 48% of control) and 3/30 (10.3 ± 2.8 nmol/mg/20 min; 42% of control) when compared to control clones (25.4 ± 1.9 nmol/mg/20 min and 24.0 ± 1.6 nmol/mg/20 min). Thus the two clones 3/29 and 3/30, with the highest level of α_{i3} expression, accumulated cAMP at less than half the rate of control cells. A third clone, 3/28, with an intermediate level of α_{i3} expression, demonstrated a smaller decrease in cAMP accumulation than clones 3/29 and 3/30. In contrast, transfection of α_{i3}-Q204L had no significant effect on forskolin-induced cAMP accumulation in clones 3/22, 3/24 and 3/27, which have low to intermediate levels of α_{i3} expression.

3.3. *Cellular localization of α_{i3} in NIH 3T3 clones expressing α_{i3}-Q204L*

When control cells were examined by whole cell fluorescence microscopy, we found α_{i3} to be restricted to a

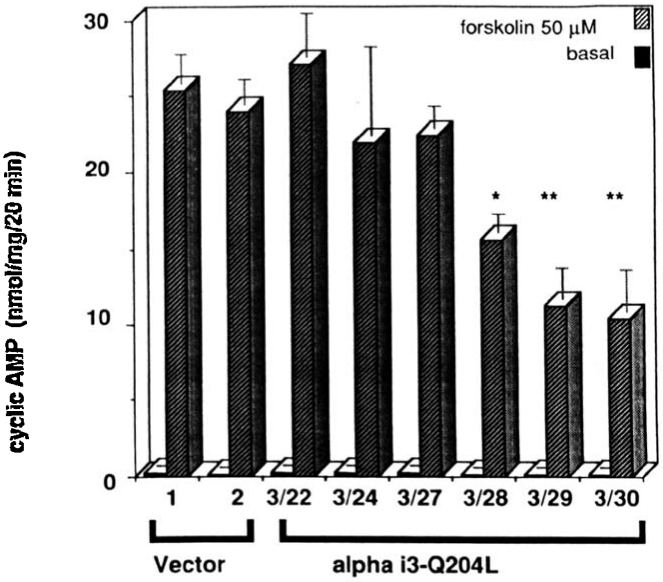

Fig. 2. cAMP accumulation in NIH 3T3 clones expressing α_{i3}-Q204L. NIH 3T3 clones were incubated with or without forskolin (50 μM) at 37°C for 20 min. Values are mean ± S.D. of three different experiments performed as described in section 2. $^{*}P < 0.05$; $^{**}P < 0.01$.

prominent perinuclear network that is typical of the Golgi apparatus in these fibroblasts (Fig. 3a). To confirm the apparent lack of plasma membrane staining, we also examined semithin (0.5–1.0 μm) cryosections of control cells. This technique of staining is useful to distinguish membrane labeling from potential cytosolic pools. By this technique, α_{i3} is undetectable on the plasma membrane of control cells and a strong signal is seen only in the Golgi region, consistent with the pattern seen in whole cells (Fig. 3b).

We next examined the six NIH 3T3 clones transfected with α_{i3}-Q204L cDNA. In all cases, the overall fluorescence of these clonal populations was higher than in the control cells and it was necessary to decrease the concentration of EC antibody 4-fold in order to clearly distinguish membrane associations from cytosolic pools in whole cell immunofluorescence. As shown in Fig. 4a–f, four of the clones (3/22, 3/24, 3/27 and 3/28) continue to have a predominant Golgi signal for α_{i3}, despite a clear increase in α_{i3} expression. Staining can occasionally be seen at the cell periphery in these clones (arrowhead, 3/24) but is not typical of the majority of cells. The two clones with very high levels of expression, 3/29 and 3/30, are dramatically different when examined by whole cell fluorescence microscopy. Specific staining for α_{i3} is clearly demonstrable at the plasma membrane of the majority of cells in these clonal populations, and is particularly striking in membrane ruffles and at the leading edge of filipodia. Thus, the two clones with the highest level of expression, 3/29 and 3/30, have high levels of plasma membrane association for activated α_{i3} and, correspondingly, show a clear inhibition of cAMP accumulation. Three clones with low to moderate overexpression of activated α_{i3} (clones 3/22, 3/24, 3/27) maintain the Golgi association seen in control cells, do not demonstrate association of α_{i3} with the plasma membrane and do not show any change in cAMP accumulation. One clone (3/28) appeared to be an anomaly in this respect: plasma membrane association was not clearly evident in whole cell fluorescence, but cAMP accumulation was consistently inhibited in these cells, although not as well as in clones 3/29 and 3/30. Therefore we compared localization of α_{i3} in semithin sections of clone 3/28 with other moderate (3/22) and high (3/29) overexpressing clonal populations. As shown in Fig. 5a–c, a typical Golgi signal (and no 'rim' signal) is seen for clone 3/22 (which does not inhibit forskolin-induced cAMP increase), while a strong 'rim' signal indicating plasma membrane association is seen for virtually every

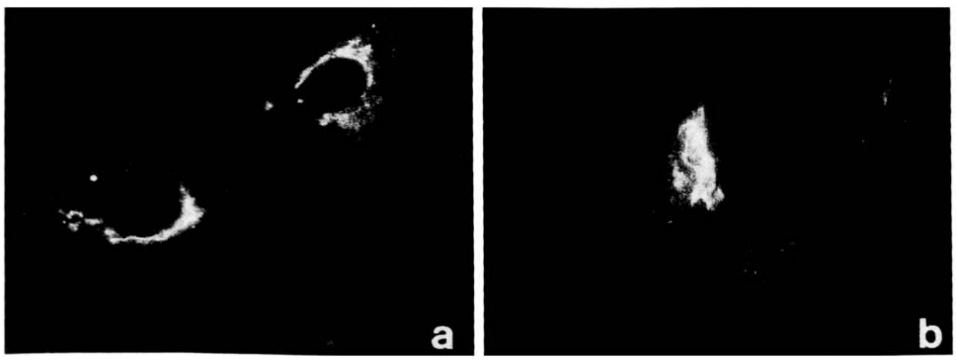

Fig. 3. Immunofluorescence localization of the α subunit of G_{i3} in NIH 3T3 cells transfected with vector alone (clone 4/6). (a) Cells grown on coverslips, fixed with 2% paraformaldehyde, permeabilized with 1% Triton X-100, and stained for whole cell fluorescence. (b) Cryosection (1 μm thick) of cells fixed in 2% formaldehyde/75 mM lysine/10 mM periodate and stained for fluorescence. Anti-α_{i3} antiserum (EC) was used at a dilution of 1:400, followed by FITC-conjugated donkey anti-rabbit IgG. Via both methods, G_{i3} is shown to be targeted solely to the Golgi region adjacent to the nucleus.

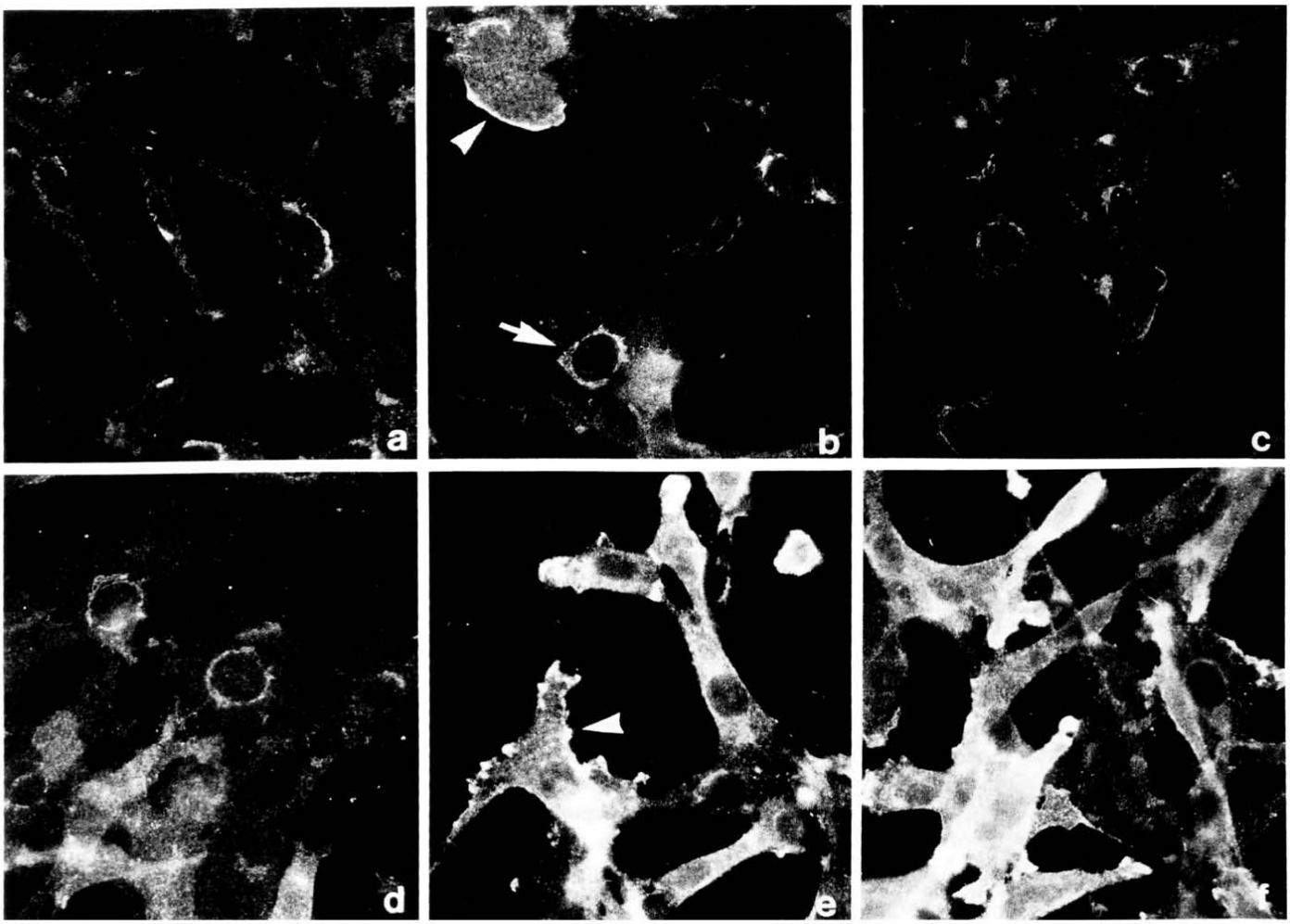

Fig. 4. Whole cell immunofluorescence localization of G_{i3}. Whole cell immunofluorescence localization of G_{i3} in cells expressing low ((b), clone 3/24), intermediate ((a), clone 3/22; (c) clone 3/27; (d) clone 3/28) and high ((e), clone 3/29; (f) clone 3/30) levels of α_{i3}-Q204L. Cells grown on coverslips were fixed with 2% paraformaldehyde, permeabilized with 1% Triton X-100 and labeled with anti-α_{i3} antiserum (EC) at a dilution of 1:1600, followed by FITC-conjugated donkey anti-rabbit IgG. Low to moderate overexpression of α_{i3} (a–d) results in staining typical of Golgi localization (as in (b), arrow), although some plasma membrane association can be found in a minority of the cells ((b), arrowhead). High levels of overexpression of α_{i3} (e,f) are shown to result in intense staining of the plasma membrane; arrowhead in (e) marks example of a membrane ruffle that is brightly labeled.

cell from the population originating from clone 3/29 (which effectively inhibits cAMP accumulation). The staining pattern for clone 3/28 is more heterogenous, but a significant number of cells do show the rim-like stain of the plasma membrane (Fig. 5b, arrowhead), in addition to an intracellular pattern consistent with the Golgi localization (Fig. 5b, arrow).

4. DISCUSSION

We studied the localizatioan of G_{i3} in normal NIH 3T3 cells and found that α_{i3} subunits are targeted preferentially to the Golgi, with minimal (if any) expression at the plasma membrane. Because of the limitations of the immunofluorescence technique used for cellular localization, it is possible that α_{i3} is present at the plasma membrane in NIH 3T3 cells but at levels too low to be detected even in one μm semithin sections. However, stably transfected NIH 3T3 clones overexpressing activated α_{i3} demonstrated either predominant Golgi localization of α_{i3} (clones 3/22, 3/24, 3/27), or expression of α_{i3} at both Golgi and plasma membranes (clones 3/28, 3/29, 3/30). These latter clones are the only ones for which there is evidence of inhibition of forskolin-induced cAMP accumulation. Although it has been reported that activated α_{i3} inhibits adenylyl cyclase activity in pools of stably transfected NIH 3T3 cells [12], this does not happen in more physiological conditions, such as low or moderate expression of activated α subunits. Therefore, our conclusion, in agreement with McClue et al. [32], is that activation of α_{i3} does not induce inhi-

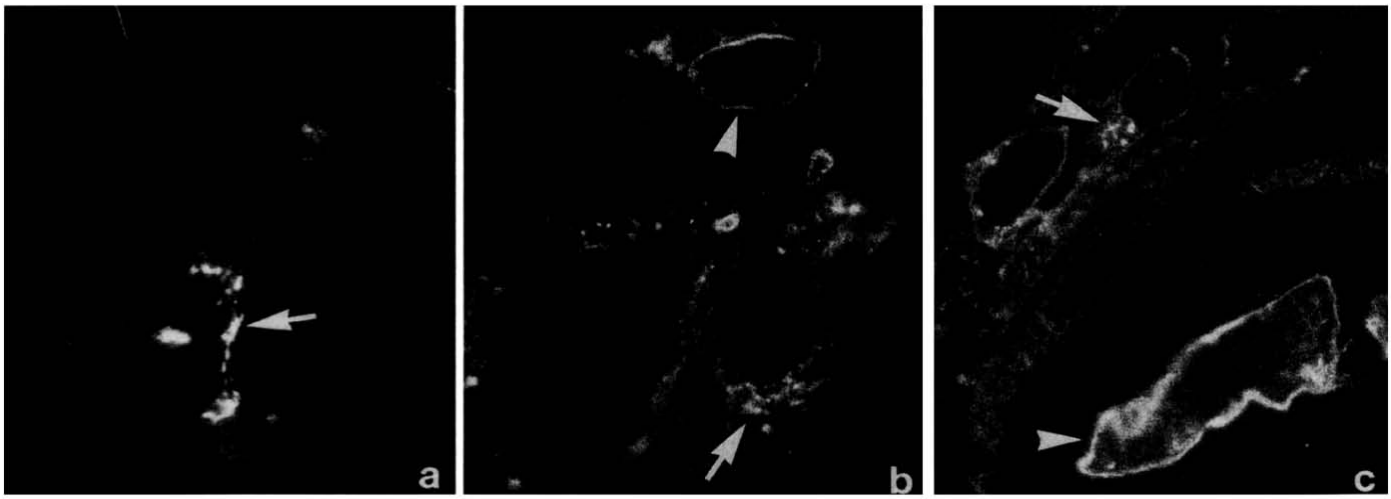

Fig. 5. Semithin cryosections of clones 3/22, 3/28 and 3/30 stained for fluorescence with the EC antibody. Clones 3/22 (a) and 3/28 (b) both express moderate levels of α_{i3}, but only in clone 3/28 (b) can both Golgi (arrow) and plasma membrane (arrowhead) localization be consistently demonstrated. Clone 3/22 is labeled only in the Golgi region next to the nucleus (a). Clone 3/30 (c) expresses high levels of activated α_{i3} and the majority of cells are intensely labeled at both the plasma membrane ((c), arrowhead) and at the perinuclear Golgi region ((c), arrow).

bition of adenylyl cyclase in normal fibroblasts. Concentrations of α_{i3} at the plasma membrane, if α_{i3} does exist at levels below our detection, are extremely low in normal fibroblasts.

It is now apparent that G_i proteins have variable subcellular localizations; therefore, their functions are probably not limited to the coupling of plasma membrane receptors to their effectors. α subunits of G_s and G_{il} have been reported to bind to tubulin [16]. Besides the plasma membrane, α_i subunits are present in specific granule fractions derived from neutrophils [14,33], in sarcoplasmic reticulum fractions prepared from canine heart [19], in rough endoplasmic reticulum of canine pancreas [18] and possibly in mitochondria (B.S. Wilson and M. Gist Farquhar, unpublished observations) [15]. Our experience with stable transfectants of α_{i3} suggests that, when expressed in large quantity, α subunits can lose the specificity of their localization in the cell. Specific binding sites for α_{i3} in the Golgi in NIH 3T3 cells can apparently be saturated and excess α_{i3} subunits targeted to the plasma membrane. At the plasma membrane, α_{i3} is able to interact with effector enzymes, mimicking biochemical actions of other closely related α subunits. Under these conditions, α_{i3} can modify adenylyl cyclase activity, probably by substituting for α_{i2}. Because membrane reconstitution systems and transfection and overexpression in mammalian cells are two very common ways of studying the functions of G proteins, our results underline the necessity to determine the exact localization in the cell of the different α subunits of G proteins, so that they can be studied in their normal environment. In recent experiments screening a large number of cell types for the distribution of α_{i3} subunits, we have found other examples of apparent exclusive Golgi distribution. Included in this category are NRK cells, REF-52 cells and murine erythroleukemia (MEL) cells (B.S. Wilson and M. Gist Farquhar, unpublished observations). We have also noted examples of cells with both Golgi and plasma membrane α_{i3} localization (Wistar rat thyroid cells) and at least one case with an apparent exclusive plasma membrane association for α_{i3} (CHO cells).

Thus, α_i subunits, despite their high sequence homology, can have distinct localization in the cell. Furthermore, the distribution of α_{i3} seems to be determined by factors specific to a given cell type. Such specificity may be defined in part by the endogenous levels of expression of α subunits and the composition and availability of membrane receptors and effectors at specific subcellular locations. Posttranslational modifications, possibly different from one cell type to another, or variable affinity or specificity for $\beta\gamma$ subunits [34], may also determine membrane targeting, subcellular localization, and, finally, functions of the α subunits of G proteins. We conclude that, until more is known about G protein cellular localization, one should be cautious in interpreting findings about G protein functions using overexpression of G protein subunits in mammalian cells.

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REFERENCES

[1] Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) Nature 348, 125–132.
[2] Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) Nature 349, 117–127.

[3] Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science 252, 802–808.
[4] Spiegel, A.M., Shenker, A. and Weinstein, L.S. (1992) Endocr. Rev. 13, 1–30.
[5] Wilkie, T.M., Gilbert, D.J., Olsen, A.S., Chen, X.N., Amatruda, T.T., Korenberg, J.R., Trask, B.J., De Jong, P., Reed, R.R., Simon, M.I., Jenkins, N.A. and Copeland, N.G. (1992) Nature Genetics 1, 85–91.
[6] Birnbaumer, L. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 675–705.
[7] Jones, D.T. and Reed, R.R. (1987) J. Biol. Chem. 262, 14241–14249.
[8] Lochrie, M.A. and Simon, M.I. (1988) Biochemistry 27, 4957–4965.
[9] Cui, Z., Zubiaur, M., Bloch, D.B., Michel, T., Seidman, J.G. and Neer, E.J. (1991) J. Biol. Chem. 266, 20276–20282.
[10] Hermouet, S., Merendino Jr., J.J., Gutkind, J.S. and Spiegel, A.M. (1991) Proc. Natl. Acad. Sci. USA 88, 10455–10459.
[11] Lowndes, J.M., Gupta, S.K., Osawa, S. and Johnson, G.L. (1991) J. Biol. Chem. 266, 14193–14197.
[12] Wong, Y.H., Conklin, B.R. and Bourne, H.R. (1992) Science 255, 339–342.
[13] Wong, Y.H., Federman, A., Pace, A.M., Zachary, I., Evans, T., Pouysségur, J. and Bourne, H.R. (1991) Nature 351, 63–65.
[14] Rotrosen, D., Gallin, J.I., Spiegel, A.M. and Malech, H.L. (1988) J. Biol. Chem. 263, 10958–10964.
[15] Lewis, J.M., Woolkalis, M.J., Gerton, G.L., Smith, R.M., Jarett, L. and Manning, D.R. (1991) Cell Regul. 2, 1097–1113.
[16] Wang, N. and Rasenick, M.M. (1991) Biochemistry 30, 10957–10965.
[17] Bokoch, G.M., Bickford, K. and Bohl, B.P. (1988) J. Cell Biol. 106, 1927–1936.
[18] Audigier, Y., Nigam, S.K. and Blobel, G. (1988) J. Biol. Chem. 263, 16352–16357.
[19] Scherer, N.M., Toro, M., Entman, M.L. and Birnbaumer, L. (1987) Arch. Biochem. Biophys. 259, 431–440.
[20] Stow, J.L., Sabolic, I. and Brown, D. (1991) Am. J. Physiol. Renal, Fluid Electrolyte Physiol. 261, F831–F840.
[21] Stow, J.L., De Almeida, J.B., Narula, N., Holtzman, E.J., Ercolani, L. and Ausiello, D.A. (1991) J. Cell Biol. 114, 1113–1124.
[22] Barr, F.A., Leyte, A., Mollner, S., Pfeuffer, T., Tooze, S.A. and Huttner, W.B. (1991) FEBS Lett. 294, 239–243.
[23] Donaldson, J.G., Kahn, R.A., Lippincott-Schwartz, J. and Klausner, R.D. (1991) Science 254, 1197–1199.
[24] Ktistakis, N.T., Linder, M.E. and Roth, M.G. (1992) Nature 356, 344–346.
[25] Jainchill, J.L., Aaronson, S.A. and Todaro, G.J. (1969) J. Virol. 4, 549–553.
[26] Nakamaye, K.L. and Eckstein, F. (1986) Acta Chir. Scand. 14, 9679–9698.
[27] Cepko, C.L., Roberts, B.E. and Mulligan, R.C. (1984) Cell 37, 1053–1062.
[28] Wigler, M., Silverstein, S., Lee, L.S., Pellicer, A., Cheng, Y.C. and Axel, R. (1977) Cell 11, 223–232.
[29] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[30] Simonds, W.F., Goldsmith, P.K., Codina, J., Unson, C.G. and Spiegel, A.M. (1989) Proc. Natl. Acad. Sci. USA 86, 7809–7813.
[31] Harper, J.F. and Brooker, G. (1975) J. Cyclic Nucleotide Res. 1, 207–218.
[32] McClue, S.J., Selzer, E., Freissmuth, M. and Milligan, G. (1992) Biochem. J. 284, 565–568.
[33] Volpp, B.D., Nauseef, W.M. and Clark, R.A. (1989) J. Immunol. 142, 3206–3212.
[34] Kleuss, C., Scherübl, H., Hescheler, J. and Schultz, G. (1992) Nature 358, 424–426.