Immunolocalization of G α i-3 in foetal lung fibroblasts

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ABSTRACT: Guanosine triphosphate (GTP)-binding proteins or G proteins are involved in a wide variety of well-recognized signalling activities between cell surface receptors and effectors. The heterotrimeric G proteins have α , β and γ subunits organized in a trimeric structure. The aim of this study was to localize G α i-3, an important heterotrimeric G protein, in foetal lung cells.

Using a foetal lung fibroblast cell line (RFL-6), the localization of G α i-3 was determined by immunofluorescence using a specific antibody to G α i-3, colocalization with a lectin known to bind the Golgi complex and Western blotting of RFL-6 cellular membrane proteins.

This study identified G α i-3 on the Golgi membranes in rat foetal lung cells. Treatment with cycloheximide, to block protein synthesis, diminished the cytosolic distribution of the protein, but intense Golgi staining remained. G α i-3, therefore, appears to be part of the Golgi complex and not present transiently during protein synthesis.

In the nonpolar foetal lung fibroblasts studied, the intracellular concentration of G α i-3 suggests a role for this protein in the intracellular trafficking and regulation of proteins needed for normal lung development.

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The normal development of the lung is dependent on signal transduction and communication within and between cells. A major family of proteins known as guanine nucleotide regulatory proteins (G proteins) is used in translating extracellular signals into intracellular events [1–3]. In particular, the high molecular weight G proteins, which are heterotrimers composed of α , β and γ subunits, have been traditionally associated with transduction at the cell surface, rather than with intracellular activities, such as constitutive protein secretion [4]. These subunits have been conserved in eukaryotes throughout evolution. Functionally, G proteins can be classified as G $_s$, activated by cholera toxin, or G $_i$, inhibited by pertussis toxin. The family of guanosine triphosphate (GTP)-binding proteins has been expanded to include proteins involved in secretion [5].

Low molecular weight G proteins, such as rab6p, have been associated with the Golgi complex and identified in the regulation of vesicular trafficking and constitutive protein secretion [6]. Heterotrimeric G α i-3 has also been found on the Golgi of polarized mature renal epithelial cells [7]. The topographic segregation of the G α i-3 subunit in foetal lung fibroblasts may suggest a functional intracellular role for the α subunit during lung development.

Pertussis toxin-sensitive G α i-3 could play an important role in the foetal lung by regulating Golgi trafficking and vesicular protein transport. The present study was conducted to determine the association of G α i-3 with Golgi complex membranes in rat foetal lung fibroblasts (RFL-6).

Materials and methods

Cell culture

RFL-6 (American Type Culture Collection, Rockville, MD, USA) is a cell line derived from the foetal lung of Sprague–Dawley rats. The cells were grown and maintained in Hams F12K medium (Gibco, Washington, DC, USA) containing 20% foetal bovine serum, 2% glutamine, 100 U·mL⁻¹ penicillin and 100 U·mL⁻¹ streptomycin at 37°C in 5% CO₂/95% air. After growth to an adherent monolayer, at days 3–5 after passage, when G α i-3 expression is maximal, the cells were treated with 0.1% trypsin–ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) and detached. The medium containing serum was used to deactivate the trypsin. The cells were then grown on sterile glass coverslips and used after 3–5 days.

EC antibody characterization

The EC antibody (Dupont, Boston, MA, USA) binds with specificity to the C-terminus of the G α i-3 subunit. The term EC refers to the original clone of G α i-3. The EC antibody has some cross-reactivity with G $_o$, which is not found in rat foetal lung cells [8]. The antibody is a polyclonal rabbit antibody and is supplied as rabbit antisera (New England Nuclear & Dupont, Boston, MA, USA). It has been confirmed, by the supplier, to bind to the G α i-3 subunit at a 1:1,000 dilution in Western blotting.

Immunocytochemistry

Cells were fixed with methanol:acetic acid (3:1), permeabilized with 0.1% Triton-X100 and then incubated sequentially with EC antibody and goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) (Kirkegaard and Perry, Gaithersburg, MD, USA). $G\alpha i-3$ and wheatgerm agglutinin (WGA)-rhodamine (Vector Laboratories, Burlingame, CA, USA) were colocalized by double labelling the cells. The negative control staining was performed with nonimmune serum and another control was not treated with any primary antibody. WGA is a well-recognized marker of the Golgi complex [7]. Cells were viewed by conventional epifluorescence microscopy on a Nikon Microphot Microscope.

Cycloheximide treatment

Cells were pretreated with $10 \mu\text{g}\cdot\text{mL}^{-1}$ cycloheximide (Sigma, St Louis, MO, USA) and incubated at 37°C for 24 h. The cells were fixed and incubated with EC and goat antirabbit IgG-FITC under the same conditions as the control cells.

Membrane preparation

Adherent cells were rinsed in cold PBS and 3–5 mL homogenization buffer (0.25 mM sucrose, 10 mM tris(hydroxymethyl) aminomethane (Tris), 1 mM EDTA, pH 7.4) was added to each dish. The cells were then scraped and homogenized. The nuclear pellet was discarded after the cells were centrifuged at $1,000\times g$ for 10 min at 4°C . The supernatant was then centrifuged at $100,000\times g$ for 2 h at 4°C . This supernatant (cytosol) and the pellet (membrane) were stored at -80°C and a small aliquot from each was used for protein determination by the BioRad standard protein assay method (Hercules, CA, USA).

Western blotting

Aliquots of RFL-6 cell membrane and cytosolic proteins were solubilized by boiling in sample buffer (1% sodium dodecyl sulphate (SDS), 30 mM Tris, pH 6.8, 12% glycerol). After protein separation on a 10% SDS polyacrylamide gel, the proteins were then transferred to an Immobilon Membrane (Millipore Continental Water Systems, Bedford, MA, USA). The membrane was stained with Ponceau S in buffer to ensure equal transfer of the proteins. The destained membrane was then blocked in Superblock blotting buffer (Pierce, Rockford, IL, USA). Immunoblotting was performed with the EC antibody, to detect $G\alpha i-3$, followed by alkaline phosphatase-conjugated secondary antirabbit IgG antibody. Developing solution was used according to the assay protocol (BioRad). Alternatively, the membrane was processed in a high-sensitivity, nonradioactive chemiluminescent detection system (ECL; Amersham, Little Chalfont, UK). An autoradiograph of the immunoblot was then obtained.

Results

The $G\alpha i-3$ subunit was localized by immunofluorescence staining with EC in the RFL-6 cell line. $G\alpha i-3$ was shown to have a distinct pattern of distribution in the RFL-6 cell line. Intense fluorescence was present in a perinuclear crescent-shaped distribution, which is characteristic of Golgi complex staining (fig. 1). The negative control cells showed no such staining pattern. When the cells were sequentially treated with WGA, the $G\alpha i-3$ staining colocalized to the Golgi. WGA has been used previously as a marker for the Golgi complex [7]. The WGA staining was more pronounced in the cytoplasm than was $G\alpha i-3$ -specific staining. The perinuclear colocalization corresponds to the Golgi complex in cells treated with both EC and WGA. WGA preferentially binds *N*-acetylglucosamine and sialic acid moieties and is thus more widely distributed intracellularly than $G\alpha i-3$ (fig. 2). When RFL-6 cells were treated with cycloheximide to stop protein synthesis, there was persistent intense perinuclear staining of the Golgi complex with EC antibody. The identification of Golgi staining specific for $G\alpha i-3$ supports the finding that $G\alpha i-3$ is a resident Golgi protein, rather than a transient protein being synthesized and processed in the Golgi complex (fig. 3). All immunocytochemistry was repeated in four separate experiments. Western blotting with EC antibody confirmed the presence of the $G\alpha i-3$ 41 kDa subunit on the membranes of RFL-6 cells (fig. 4). The band corresponding to $G\alpha i-3$ was readily detectable and this result was confirmed in three separate experiments, by both alkaline phosphatase conjugation and ECL methods.

Discussion

The present study establishes for the first time the location of $G\alpha i-3$, a pertussis toxin-sensitive heterotrimeric G protein subunit, on the intracellular membranes of cultured rat foetal lung cells and its topographic concentration implies a role in Golgi trafficking and regulation of

Fig. 1. – Immunocytochemical localization of $G\alpha i-3$ in RFL-6 cells. The cells were incubated with anti- $G\alpha i-3$ peptide antibody followed by a secondary antibody, goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate. The areas of intense fluorescence are perinuclear and compact (arrow). This is an immunofluorescence pattern characteristic of Golgi complexes. (Internal scale bar = $10 \mu\text{m}$.)

Fig. 2. – Colocalization of $G\alpha i-3$ and wheatgerm agglutinin to the Golgi complex of cultured foetal rat lung cells. A) RFL-6 cells were incubated with anti- $G\alpha i-3$ antibody followed by a secondary antibody, goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate. Intense perinuclear staining (arrow) is demonstrated, as well as punctate cytoplasmic staining. B) The same cells were then sequentially incubated with wheatgerm agglutinin conjugated to rhodamine. The lectin staining is both perinuclear and cytoplasmic. The pattern of perinuclear Golgi complex staining is colocalized with $G\alpha i-3$. The arrow in B) shows an area that is not associated with the Golgi and is not colocalized. (Internal scale bar=10 μm .)

constitutive protein secretion in the developing lung. The close association between protein intracellular localization and function has been reported previously [9–11]. While this study did not determine the precise various potential functions of $G\alpha i-3$, its intracellular localization has implications for such future studies.

In RFL-6 cells, $G\alpha i-3$ was predominantly present on the Golgi complex and appeared less prominently in a punctate diffuse cytoplasmic distribution. Cycloheximide-treated cells also have the characteristic perinuclear crescent-shaped staining pattern, indicating that $G\alpha i-3$ is not merely transiently present in the Golgi complex, but rather is part of the complex itself. Colocalization of $G\alpha i-3$ with an already established marker for the Golgi complex provides further evidence that the immunolocalization of the protein is consistent with a Golgi complex distribution.

The $G\alpha i-3$ subunit was also identified by Western blotting of the RFL-6 membrane proteins. The 41 kDa pro-

Fig. 3. – Effect of cycloheximide on $G\alpha i-3$ localization. A) Perinuclear and cytoplasmic fluorescent staining in RFL-6 cells incubated with EC antibody. B) After incubation with cycloheximide to stop protein synthesis, the immunolocalization is still concentrated in the Golgi complex. (Internal scale bar=10 μm .)

tein, identified by the EC antibody, corresponds to the molecular weight of $G\alpha i-3$ [12, 13]. The exact mechanism of topographic targeting of G protein subunits to different intracellular domains, however, remains largely unknown [14].

Extracellular signals, such as hormones and growth factors, all utilize signal transduction across the cell surface to communicate with an intracellular effector [8]. This process is vital to the existence of the cell–cell differentiation and the development of an organ. Heterotrimeric G proteins are classically involved in ion channel-regulation and receptor–effector coupling across cell membranes. There is evidence that $G\alpha i-3$ may be a regulator of Golgi trafficking and constitutive protein secretion [9, 10].

Another possible function of $G\alpha i-3$ is the regulation of Golgi breakdown and reassembly during mitosis. This process ensures partitioning of the Golgi complex into each daughter cell. One study has followed the spatial distribution of Golgi membranes in the early stages of embryogenesis [15]. The identification of the amounts

Fig. 4. — Western blot analysis of RFL-6 cellular membranes and cytosol. Each lane was loaded with equal amounts of cytosolic (A) and membrane (B) protein. There is a distinct band of 41 kDa in lane B after incubation with EC antibody, which corresponds to the $G\alpha_i-3$ subunit.

and functions of the many G proteins present during cell differentiation and organ development is a future challenge. Further understanding of how the α_i gene is controlled, possible alternative splicing of $G\alpha_i-3$ messenger ribonucleic acid and how individual subunits come to have distinct intracellular locations will advance the understanding of the role of G proteins during development.

Other studies have localized heterotrimeric G proteins to the Golgi complex in mature specialized cells [9, 10], but the localization in an undifferentiated foetal lung cell line had not been previously demonstrated. The role of the heterotrimeric G proteins in the regulation of protein secretion in the developing lung will be the subject of future study. This study establishes the presence of $G\alpha_i-3$ on the Golgi, which represents a novel intracellular location for a heterotrimeric G protein in a nonpolar foetal cell line. Its localization further suggests many possible regulatory roles in the development of the normal lung.

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