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Impairment of Bile Salt-Dependent Lipase Secretion in Human Pancreatic Tumoral SOJ-6 Cells

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Abstract Bile salt-dependent lipase (BSDL) was detected in human SOJ-6 and rat AR4-2J pancreatic cells. Whereas AR4-2J cells actively secreted the enzyme, BSDL was retained within the Golgi compartment of SOJ-6 cells. Because Rab6 is involved in vesicle transport in the Golgi apparatus and the *trans*-Golgi network, we confirmed the presence of Rab6 in these cells. In rat AR4-2J cells, Rab6 as well as Rab1A/B and Rab2, partitioned between the cytosol and microsomes. In SOJ-6 cells Rab1A/B and Rab2 also partitioned between the cytosol and microsomes, but Rab6 was strictly associated with microsome membranes, suggesting a specific defect of Rab6 cycling in human SOJ-6 cells. The apparent defect of cycling in these cells is not due to the expression of a defective Rab6 since its correct sequence was confirmed. We further demonstrated that AR4-2J and SOJ-6 cells express the Rab-GDIβ and Rab-GDIα isoforms, respectively. However, the sequence of Rab-GDIβ, which may be the main form expressed by SOJ-6 cells, identified a few substitutions located in regions that are essential for Rab-GDI function. We conclude that the deficient secretion of BSDL by SOJ-6 cells could be due to the expression of defective Rab-GDIβ. In spite of the alterations in Rab-GDIβ, membrane proteins such as CD71 and NHE3 were correctly localized to the cell plasma membrane of SOJ-6 cells, suggesting that two functional distinct secretory pathway coexist in pancreatic cells. J. Cell. Biochem. 79:628–647, 2000.†

Key words: Bile salt-dependent lipase; Rab-GDI; pancreas (cancer); SOJ-6; AR4-2J (cells)

Marked secretory abnormalities are observed in the profiles of patients with cancer of the pancreas [Gordis and Gold, 1993]. In many cases, the pancreas has become almost nonfunctional and tumor cells do not secrete pancreatic enzymes. Interestingly, the pancreatic secretory profile of hamsters receiving a single injection of tumor promotor such as derivative of nitrosamine to induce pancreatic cancer exhibit similar changes in secretory profiles even before tumors can be detected [Rinderknecht et al., 1983].

Abbreviations used: BSDL, bile salt-dependent lipase (EC. 3.1.1.13); ER, endoplasmic reticulum; GAP, GTPase activating protein; Rab, Ras-like in rat brain; Rab-GDI, Rab guanine nucleotide dissociation inhibitor; Rab-GEF, Rab guanine nucleotide exchange factor; TGN, trans-Golgi network.

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The bile salt-dependent lipase (BSDL, E.C 3.1.1.13) is found in the pancreatic secretions of all species examined up to now, from fish to human [Gjellesvik et al., 1994; Lombardo et al., 1978]. This enzyme plays an essential role in the intestinal processing of cholesterol [Lombardo and Guy, 1980; Howles et al., 1996; Shamir et al., 1995]. We have shown that human [Bruneau et al., 1995] and rat BSDL [Bruneau and Lombardo, 1995], in contrast to other secretory pancreatic enzymes, are associated with intracellular membranes during the secretory process. Its release from membranes occurs in the *trans*-Golgi compartment after completion of glycosylation [Bruneau and Lombardo, 1995] and phosphorylation [Pasqualini et al., 1997]. It has been postulated that BSDL expressed by SOJ-6 cells is sequestrated within the endoplasmic reticulum (ER) [Roudani et al., 1994; Miralles et al., 1993]. Nevertheless, this protein carries out the *J28* oncofetal epitope [Mas et al., 1993] which is fucose-dependent [Panicot et al., 1999] and associated with *O*-linked glycans of BSDL [Panicot et al.,

1999; Mas et al., 1997]. Because fucose residues are added to carbohydrate structures in the medial or *trans*-Golgi cisternae [Colley, 1997], this observation suggests that BSDL reaches this compartment. Therefore, a reasonable proposal is that a disruption of a *post*-Golgi event may result in the impairment of BSDL secretion by human tumor SOJ-6 cells. This could result from, for example, a defect of the vectorial transport of cargo-vesicles in the Golgi apparatus. In contrast, BSDL is normally secreted by the rat pancreatoma AR4-2J cell line [Abouakil et al., 1993].

Rab proteins (Rabp) are Ras-like GTP binding proteins involved in the regulation of vesicular transport and about 40 members of the Rab family have been identified. Rabp proteins are specifically localized on different organelles and transport vesicles, and each membrane trafficking step appears to require a unique Rabp [Simons and Zerial, 1993]. In particular, Rab6 is found associated with medial and *trans*-Golgi cisternae as well as *post*-Golgi organelles, possibly regulating intra-Golgi transport events [Goud et al., 1990; Antony et al., 1992; Mayer et al., 1996]. Rabp interconvert between inactive GDP-bound and active GTP-bound conformations and switch between a cytosolic and membrane localization. At steady state, a given Rabp is mainly membrane-associated but a fraction can also be detected in cytosol [Goud et al., 1990]. This latter fraction represents Rabp bound to a family of proteins termed Rab-GDI (for Rab GDP-dissociation inhibitor) [Sasaki et al., 1990]. Following vesicle fusion with its target, Rab-GDI retrieves Rabp from their fusion membrane targets in their GDP-bound conformations. Rab-GDI can also deliver Rabp to their specific membrane-bound compartments to enable them to function in vesicle docking processes [Pfeffer et al., 1995]. Membrane targeting is consequently accompanied by the release of Rab-GDI, and the membrane-associated Rabp exchanges its bound GDP for GTP, a reaction likely promoted by a guanine nucleotide exchange factor (GEF). The release of Rabp from Rab-GDI involves a membrane-associated protein factor referred to as GDI-displacement factor (or GDF) [Dirac-Svejstrup et al., 1997]. This cycle, which is critical for Rabp function [Riederer et al., 1994], is likely to control vesicle targeting and fusion of the donor vesicle with the acceptor compartment in the exocytic pathway. In this study, we attempted to uncover the origin of the defect in BSDL secretion processes observed in SOJ-6 cells, focusing on Rab6 and its companion proteins. We show that, unlike AR4-2J cells which express Rab-GDIα, SOJ-6 tumoral cells express a defective form of Rab-GDIβ. This deficiency may account for an observed defect in Rab6 cycling and thus the retention of BSDL in these cells. These results indicate that the secretion pathway leading to the release of BSDL by pancreatic acinar cells is dependent upon Rab6 and Rab-GDI. Because targeting of transferrin receptor (CD71) and sodium/proton exchanger (NHE3) in SOJ-6 cells expressing mutated Rab-GDIβ is correct, the presence of another targeting route in pancreatic cells, independent of Rab6 cycling, is suggested.

MATERIALS AND METHODS

Reagents

RPMI-1640 and DMEM medium, glutamine, penicillin, trypsin-EDTA, and streptomycin were from Life Technologies (Bethesda, MD). Fetal calf serum (FCS) was from Dutscher (Brumath, France). β-phenyl propionate was from Fluka (Buchs, Switzerland). Nonidet P40 (NP40), Polyvinylpyrolidone K40 (PVP 40), phenylmethyl sulfonylfluoride (PMSF), benzamidine, Coomassie blue R250, FITC-conjugated anti-rabbit goat IgG, TRITC-conjugated anti-mouse goat IgG, alkaline phosphatase-conjugated anti-rabbit, and anti-mouse goat IgG, 4-nitrophenyl hexanoate, lovastatin, and Soybean Trypsin inhibitor (STI) were from Sigma (St. Louis, MO). Nitrocellulose (BA 83 type, 0.2 μm) membranes were from Schleicher and Schuell (Dassel, Germany). Recombinant Rab-GDIα was from Calbiochem (La Jolla, CA). Taq DNA polymerase and dNTPs were from Promega (Madison, WI). Avian myeloblastosis reverse transcriptase, human placenta RNase inhibitor, and RNAse A were from Roche Diagnostics (Meylan, France). pCR2.1 vector (TA-cloning) was from Invitrogen (Leek, The Netherlands). [^{35}S]-αGTP, [^{125}I]-labeled protein A were from ICN Biochemicals (Costa Mesa, CA). [2^{-14}C]-mevalonic acid lactone was from Amersham (Buckinghamshire, UK).

Biological Materials

Polyclonal antibodies (*pAb L64*) directed against human BSDL were raised as previously described [Abouakil et al., 1988] and purified on Protein A-Sepharose. Rab6 antibodies were developed in rabbit using the synthesized C-terminal peptide of Rab6. This peptide which includes Ser184 to Glu203, was covalently linked to KLH before immunization. Antibodies were then isolated by immunoaffinity on agarose-immobilized antigenic peptide. Alternatively, commercial antibodies specific for Rab6 and raised against the same peptide (Santa Cruz-Biotechnology, Santa Cruz, CA) were used without any difference in results. Rabbit polyclonal antibodies to Rab1 (A and B isoforms) and Rab2 were obtained from Zymed (San Francisco, CA) and Santa Cruz, respectively. These two antibodies are specific of the C-terminal domain of the corresponding protein and are reactive with human and murine antigens. Rabbit polyclonal antibodies to Rab-GDI-1 and mouse monoclonal antibodies to CD71 (clone My29) were from Zymed. Polyclonal antibodies specific for Rab-GDI were raised in mouse against a peptide sequence conserved in bovine, mouse, rat and human Rab3A-GDI, Rab-GDIα, and Rab-GDI-1 proteins, and cross-react with Rab-GDI-2. Monoclonal antibodies specific for the 58K-Golgi protein were from Sigma. Polyclonal antibodies to CD71 used in Western blotting were purchased from Santa Cruz. Normal and tumor human pancreatic tissues were from adult patients (females, aged 63 and 60, respectively). Immediately after resection, tissue samples were cut in small pieces, frozen in liquid nitrogen, and stored at −80°C until use.

Cell Lines and Culture

The human pancreatic adenocarcinoma SOJ-6 cells [Fujii et al., 1990] were kindly provided by Dr. Escribano (INSERM, U-260). They were grown in RPMI medium supplemented with 10% FCS, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The rat pancreatoma AR4-2J cells were obtained from Dr. Clémente (INSERM, U-151) and cultured in DMEM containing 10% FCS, glucose (1 g/L), glutamine, penicillin, and streptomycin. Cells were kept in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2.

Cell and Tissue Extracts and Fractionation

Cells grown to confluence were harvested with 0.25% trypsin-EDTA, after being rinsed twice with incomplete PBS buffer (10 mM phosphate buffer pH 7.0 with 0.15 M NaCl, without Ca^{2+} or Mg^{2+}). The cells were suspended in RPMI or DMEM (10% FCS) and centrifuged at 1,000*g* for 2 min. The cells were lyzed by sonication for 10 s (Bioblock Vibra Cell, Strasbourg, France) in PBS buffer containing the following proteases inhibitors: 0.1 μg/ml STI, 2 mM PMSF, 2 mM β-phenyl propionate, 2 mM benzamidine. The cell debris was pelleted by centrifugation (1,000*g*, 5 min) and the supernatant was centrifuged at 150,000*g* for 1 h at 4°C to separate the cytosol from the microsome membrane fraction.

Protein Determination and Enzyme Assays

The cell monolayer was washed with fresh medium and with PBS. The cellular proteins were obtained after lysis as described above and quantitated [Lowry et al., 1951]. The esterolytic activity of BSDL was measured using 4-nitrophenyl hexanoate by monitoring the change in absorbance at 404 nm and pH 7.4 in a thermostated cell at 30°C, as described elsewhere [Gjellesvik et al., 1992]. Lactate dehydrogenase activity was determined as described by Goldberg [1972].

Polyacrylamide Gel Electrophoreses and Western Blotting

Electrophoresis (SDS-PAGE) was performed on 12% (w/v) polyacrylamide gels, 0.1% sodium dodecyl sulfate as described by Laemmli [1970]. After migration, the proteins were electrophoretically transferred to nitrocellulose membrane [Burnette, 1981] in 50 mM Tris/HCl, 40 mM glycine, and 20% ethanol buffer, at 150 mA for 3 h in a cold room. The completeness of the transfer was checked by staining gels with Coomassie blue R250 and destained in ethanol/acetic acid/ water (2/3/35 per volume). Membranes were first blocked for 1 h in 50 mM Tris/HCI, pH 8.0 buffer containing 150 mM NaCl (TBS buffer) and 3% bovine serum albumin (BSA, blocking buffer). Immunodetection was carried out using *pAb L64* or antibodies specific for Rab6, Rab1A/B, Rab2, Rab-GDI, CD71, or NHE3 as primary antibody. Incubation was carried out for 1 h in the blocking buffer plus 0.1% Tween 20. Blots were then

incubated in the above buffered BSA solution containing alkaline phosphatase-conjugated anti-rabbit or anti-mouse goat IgG and developed as previously described [Mas et al., 1997] or using the BM chemiluminescence Western-blotting kit (Roche Diagnostic, Meylan, France). Alternatively, antigen-antibody complexes were detected by [^{125}I]-protein A overlay (0.25 μCi/ml of the blocking buffer, 0.1% Tween 20, 1 h at room temperature). Under these conditions, PVP 40 (2% in TBS buffer) was used instead of BSA to saturate membranes and to dilute antibodies. Blots were exhaustively washed, air-dried, and autoradiographed. Quantitation of the stained bands was performed after scanning using the IMAGE program (NIH, Bethesda, MA).

Immunoprecipitation

An aliquot of Protein A-Sepharose was pelleted by centrifugation and incubated with proteins in the presence of either *pAbL64* (5 μg) or antibodies specific for Rab6 (5 μg). Controls were performed in the absence of antibodies or in the presence of Rab6 C-terminal peptide as competitor. At the end of the incubation (usually overnight at 4°C), antigen-antibody-Protein-A complexes were recovered by centrifugation (10,000*g*, 15 min) in TNN buffer (10 mM Tris/HCl, pH 7.5; 150 mM NaCl, and 1% Nonidet P40). The pellet was washed twice with TNN buffer and twice again with 10 mM Tris/HCl, pH 7.5 buffer. The pellet was then transferred into the SDS-PAGE Leammli's sample buffer, boiled for 5 min, centrifuged, and electrophoresed.

GTP Binding Assay

The binding of GTP to Rab6 in cytosolic and membrane fractions has been studied using a [^{35}S]-αGTP-ligand assay [Escola et al., 1995; Göke et al., 1992]. Rab6 was immunoprecipitated overnight at 4°C with specific antibodies (see above). The pellets were washed and bound material was eluted by boiling samples for 5 min in SDS-PAGE sample buffer containing 5 mM DTT. The samples were electrophoresed on SDS-PAGE and gels were soaked in 50 mM Tris/HCl, pH 7.5; 20% glycerol. The last operation was repeated twice for 10 min. The proteins were then electrotransferred to nitrocellulose membranes using sodium carbonate buffer (10 mM NaHCO3, 3 mM Na2CO3, pH 8.9). After transfer, blots were rinsed twice for 10 min in the binding buffer (50 mM Tris/HCl, pH 7.5; 12 μM MgSO4; 1 mM 2-mercaptoethanol; 10 μM MgATP, and 0.3% Tween 20), and incubated in fresh binding buffer containing 2 μCi/ml [^{35}S]-αGTP for 30 min. Membranes were washed three times for 10 min in the binding buffer, air-dried, autoradiographed, and quantitated as described above.

Reverse Transcription, Polymerase Chain Reaction, cDNA Cloning, and Sequencing

The oligonucleotides used for PCR were synthesized from the cDNA sequence encoding human Rab6 [Zahraoui et al., 1989] and designed to match the entire nucleotide sequence (upstream primer, 5′-TTTCGTCCaagcttCCACAATGTCCACGGGCGGAGACTTC-3′, and downstream primer, 5′-TTTCGTggatccGGGACATTAGCAGGAACAGCCTCC–3′). *Hind*III and *Bam*H1 restriction sites were inserted in 5′ of the upstream and downstream primers, respectively (small case letters). To obtain the cDNA fragment, total RNA from SOJ-6 cells was extracted using the Chirgwin's method [Chirgwin et al., 1979] and reverse transcribed (First strand cDNA synthesis kit from Roche). The cDNA pool obtained was amplified by performing a polymerase chain reaction (PCR). The amplification reaction mixture (50 μl) consisted of 5 μl of cDNA, 600 ng of each primers, 0.2 mM dNTPs, 1.5 mM MgCl2, and 2.5 units of Taq DNA polymerase. The Rab6 cDNA was amplified for 35 cycles (GeneAmp PCR 2400 system, Perkin Elmer) with denaturation at 94°C (1.5 min), annealing at 63°C (1.5 min), and extension at 74°C (2 min). The extention was terminated by a final incubation at 74°C for 10 min.

Four pairs of primers matching the 5′ and 3′ ends of rat RabGDIα and β and of human RabGDI 1 and 2 were synthesized. They should allow us to amplify the entire mRNA sequence coding for each RabGDI according to species. The sequences of these primers are as follows, upstream primers: rat Rab-GDIα, 5′-TTTCGTAAGCTTATGGATGAGGAATACGA TGTGATT-3′; rat Rab-GDIβ, 5′-TTTCGTAAGCTTATGAATGAGGAATACGAC-3′; human Rab-GDI1, 5′-TTTCGTAAGCTTATGGACGAGGAGTACGATGT-3′; human Rab-GDI2, 5′-TTTCGTAAGCTTATGGACGAGGAATACGATGT-3′; downstream: rat RabGDIα, 5′-TTT CGTCTCGAGCACTGATCGGCTTCTCCAAAGACA-3′; rat Rab-GDIβ, 5′-TTTCGTGA

ATTCTTAGTCTTCTCCATAAATGTC-3′; human Rab-GDI1, 5′-TTTCGTGAATTCTCACTGCTCAGCTTCTCCA-3′; human Rab-GDI2, 5′-TTTCGTCTCGAGGCTGTTAGTCTTCCCCATAG-3′. Two other degenerated primers were designed to match common sequences of rat Rab-GDIα and Rab-GDIβ and human Rab-GDI1 and Rab-GDI2 [Nishimura et al., 1994] (upstream primer; 5′-CT(T,C)-ATGGC(T,C)AATGGTCAG–3′ and downstream primer, 5′-TCATTCT(T,G)(T,C)-TTGCGCTTCAT-3′). These primers cover the sequence of Rab-GDI from nucleotide 271 to nucleotide 1349. The amplification of RabGDI-related mRNAs were performed on a Robocycler Gradient 96 (Stratagene) as described above except the annealing temperature which was fixed at 53–54°C for full lenght amplification of RabGDI mRNAs or ranged from 40–46°C by a 1°C gradient step when using the degenerated pair of primers.

The cDNA fragments obtained after two independent RT-PCR experiments were subcloned into pCR2.1 vector. The two cDNA strands were sequenced [Sanger et al., 1977] using universal *M13* reverse and forward primers. β-Actin primers from Clontech, used according to the manufacturer's protocol, allowed us to obtain a cDNA probe specific for β-actin.

Alternatively, BSDL mRNA present in SOJ-6 and AR4-2J cells was quantitated by dot-blot dilutions. For this purpose, total RNA was blotted with decreasing half-dilutions on nitrocellulose membrane. Prehybridization and hybridization using specific cDNA probes for BSDL [Roudani et al., 1994] and β-actin was performed essentially as described [Pasqualini et al., 1998]. Before hybridization, probes were [^{32}P]–labeled by random-priming (Life Technologies) using [α-^{32}P] dCTP (NEN Life Science) at a specific radioactivity of 4. 10^8 cpm/μg DNA probe.

Antisense/Sense Oligonucleotides

The procedure used in this experiment was that validated by Huber et al. [1993]. Phosphorothioate oligonucleotides were designed to hybridize on a site selected to center on the initiation ATG codon (ATG-antisense) and on a non-overlapping site located immediately downstream (inner-antisense). Since the sequence of the rat Rab6 was unknown, primers were designed according to the sequence of human Rab6 [Zahraoui et al., 1989]: ATG-antisense (5′-ACATTGTGGAACTAG-3′), inner-antisense (5′-AGTCTCCGCCCGTGG-3′). A reversed ATG-antisense (5′-GATCAAGGTGTTACA-3′) was also synthesized and used as the control oligonucleotide. Oligonucleotides were added at 10 μM final concentration directly to the culture medium of 80% confluent AR4-2J cells. Cells were incubated for 70 h with either antisense (5 μM ATG-antisense and 5 μM inner-antisense) or reversed-antisense (10 μM). Fresh oligonucleotides were added every 24 h, and an aliquot of the culture medium was withdrawn and frozen. At the conclusion of the incubation period, aliquots were thawed and BSDL activity recorded using 4-nitrophenyl hexanoate.

Immunofluorescence

Cells grown on microscope slides were washed three times with incomplete PBS buffer and fixed with 3% (v/v) paraformaldehyde for 20 min. The excess paraformaldehyde was eliminated by washing the slides in 50 mM NH4Cl, and cells were permeabilised with 0.05% saponin/incomplete PBS buffer. Nonspecific sites were saturated by incubation for 30 min in 10% calf serum diluted in 0.05% saponin/incomplete PBS. The cells were further incubated for 30 min with appropriate primary antibodies. Slides were exhaustively rinsed with 0.05% saponin/incomplete PBS and incubated for 20 min with FITC-conjugated antibodies directed against rabbit IgG (10 μg/ml) or with TRITC-conjugated antibodies directed against mouse IgG (10 μg/ml). Slides were washed, first with 0.05% saponin/incomplete PBS, then with water, and mounted in 25 mM Tris/HCl, pH 8.0, 75% glycerol, and 0.1% p-phenylenediamine. Slides were examined using appropriate filter and photographed using a fluorescence microscope.

Flow Cytometry Analysis

Cells grown to 80% confluence were washed twice with PBS buffer then dissociated in 5 ml of non-enzymatic Cell Dissociation Solution (Sigma) for 15 min at 37°C. Cells were then pelleted at 1,000 rpm for 5 min at 4°C. Pellets were resuspended in 2% paraformaldehyde in 1 ml PBS and incubated 15 min at 4°C under gentle agitation. Cells were pelleted, washed in PBS, and treated with BSA (1% in PBS). They were then incubated once for 2 h at 4°C under

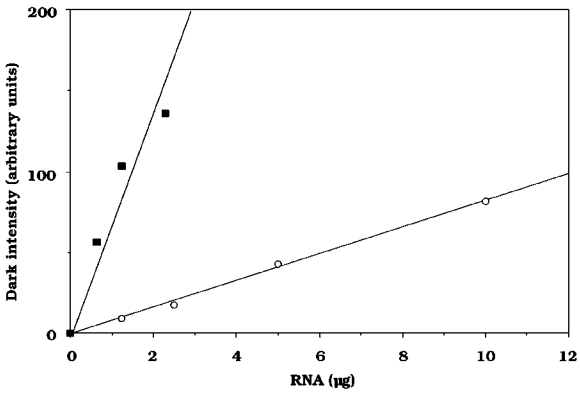

Fig. 1. Bile salt-dependent lipase mRNA in AR4-2J and SOJ-6 cells. RNA was extracted from rat pancreatic AR4-2J cells (full symbols) and from human pancreatic SOJ-6 cells (open symbols) and blot-dotted in increasing rank order of half-dilution from 10 μg down to 0.015 μg. The presence of RNA encoding BSDL was detected using radiolabeled probes, quantitated (dark intensity in arbitrary units), normalized against the actin probe is plotted as a function of the amount of RNA blotted.

gentle agitation with the appropriate dilution of primary antibodies in PBS-BSA, washed, and finally incubated for 1 h at 4°C in a dark-room with the required FITC-conjugated secondary antibodies. Cells were exhaustively washed and suspended in 150 μl of Isoflow sheath fluid (Coulter, Miami, FL) and analyzed with an Epics Profile II cytometer (Coulter).

RESULTS

Impairment of Bile-Salt-Dependent Lipase Secretion in SOJ-6 Cells

The presence of BSDL messenger RNA in SOJ-6 cells has been detected by in situ hybridization [Roudani et al., 1994] and by Northern-dot blot dilution using a probe specific for BSDL [Pasqualini et al., 1998]. Normalized to β-actin probe, this BSDL probe hybridized to mRNA in both SOJ-6 and AR4-2J cells. As illustrated in Figure 1, the amount of mRNA encoding BSDL was five to 10 times lower in SOJ-6 cells than in AR4-2J cells. Therefore, if this mRNA is translated at the same rate in each cell line, there should be five to 10 less BSDL activity expressed in SOJ-6 than in AR4-2J cells. As shown in Table I, the expression of BSDL by AR4-2J cells was efficient and after 6 h of culture, enzyme activity accumulated in the culture medium where it represents a 10-fold excess as compared to cell-associated activity. The total BSDL activity expressed by SOJ-6 cells represented some 5–10% of that expressed by AR4-2J cells and correlated with the amount of mRNA encoding BSDL. However, in contrast to AR4-2J cells, only a small amount of BSDL activity was released by SOJ-6 cells into the culture medium. This activity represented less than 15% of the total BSDL activity detectable in SOJ-6 cells. No LDH activity was found in this medium, therefore cell lysis cannot explain the presence of BSDL activity in the extracellular fraction. Thus, the secretion of BSDL by SOJ-6 cells is less efficient than that of AR4-2J cells, and it is likely that the enzyme is retained within a subcellular compartment of SOJ-6 cells.

To further characterize the retention of BSDL in SOJ-6 cells, immunofluorescence studies were performed. In SOJ-6 cells, BSDL partly co-localizes with the 58K-Golgi protein (Fig. 2A,B), however some BSDL labeling was spread out within the cytoplasm of cells which confirms the presence of the protein in the ER. The localization of BSDL within the Golgi stacks of SOJ-6 cells agrees with its reactivity with the *mAbJ28* [Miralles et al., 1993] which detects the presence of the *J28* glycotope on BSDL [Mas et al., 1993, 1997]. Human and rat pancreatic BSDL are associated with intracellular membranes during their secretory process [Bruneau et al., 1995; Bruneau and Lombardo, 1995] and the release of the enzyme occurs in the *trans*-Golgi compartment [Pasqualini et al., 1997]. Consequently, if BSDL in SOJ-6 cells reaches a compartment following the *trans*-Golgi, it should be found associated with the intraluminal soluble fraction. Indeed, in SOJ–6 cells more than 80% of the intracellular enzyme was associated with the microsome membrane pellet (Fig. 3). These data indicate that BSDL is associated with membranes and mainly retained within the Golgi compartment of SOJ-6 cells.

The retention of BSDL cannot result from inherent properties of the protein as we have shown by transfecting CHO cells with the cDNA of BSDL isolated from either SOJ-6 cells (which encode the feto-acinar pancreatic protein or FAPP [Pasqualini et al. 1998]) or normal pancreatic tissue. The levels of FAPP and BSDL which are secreted by transfected CHO cells were comparable and higher than that observed with SOJ-6 cells [Pasqualini et al., 1998]. Furthermore, we have shown that the secretion of BSDL is regulated by phosphorylation of the enzyme by a CKII protein kinase,

TABLE I. Secretion of BSDL*

Cells	Activity (10^{-3} units)		Extracellular activity
	Intracellular	Extracellular	% of total activity
AR4-2J cells	53.0 ± 6.0	526.0 ± 134.0	90.8 ± 23.1
SOJ-6 cells	29.5 ± 1.3	4.1 ± 0.7	12.2 ± 2.4

*Cells were cultured in fresh medium for 6 h. BSDL activity was then measured in cell free-medium (Extracellular activity) and cells were harvested and lysed for determination of cell-associated BSDL activity (Intracellular activity).

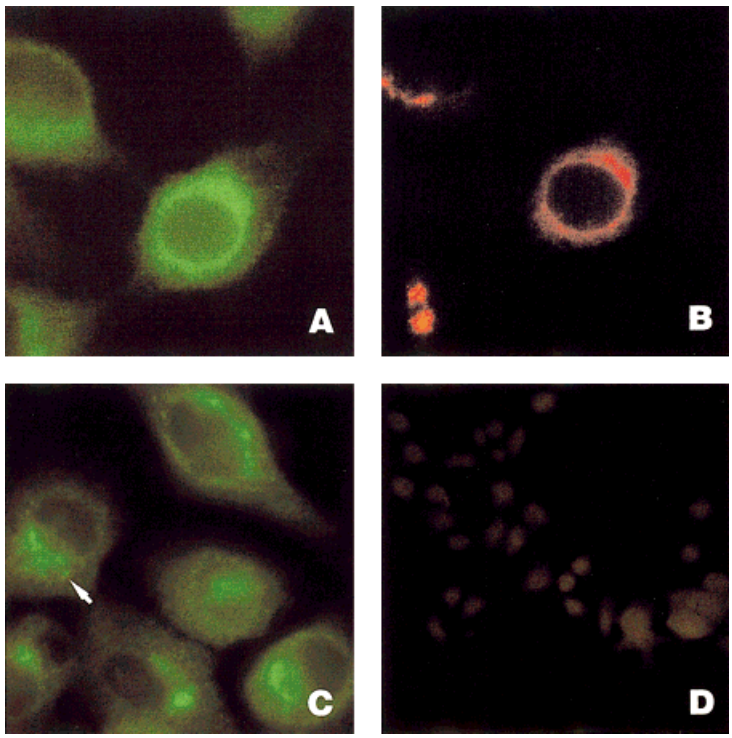

Fig. 2. Localization of BSDL and Rab6 in SOJ-6 cells. Co-localization of BSDL and 58K-Golgi protein in SOJ-6 cells. **A**: SOJ-6 cells labeled with *pAbL64* and FITC-conjugated antibodies directed against rabbit IgG. **B**: The same SOJ-6 cells treated with antibodies specific for the 58K-Golgi protein and TRITC-conjugated antibodies directed against mouse IgG. Localization of Rab6 in SOJ-6 cells. **C**: SOJ-6 cells labeled with antibodies specific for Rab6 and FITC-conjugated antibodies directed against rabbit IgG. The peptide used to raise antibodies to Rab6 abolished the reactivity of these antibodies with SOJ-6 cells antigenic sites (not shown). Control (**D**), SOJ-6 cells incubated with secondary antibodies but omitting prior incubation with primary antibodies.

likely located in the trans-Golgi compartment [Pasqualini et al., 1997, 2000]. Similarly to BSDL, FAPP is also phosphorylated (unpublished observation). Altogether these data suggest that the secretion of BSDL is defective in human tumor pancreatic SOJ-6 cells at the level of the Golgi.

Function of Rab6 in Pancreatic Cells

Because Rab6 has been suggested to be involved in the transit of proteins through the Golgi complex [Martinez et al., 1994], we investigated the presence of this small-GTP binding protein in the pancreatic cells used in this study. Antibodies specific for Rab6 detected this protein in AR4-2J cells and in SOJ-6 cells which migrated at ≈26 kDa (Fig. 4, arrow). Immunofluorescence studies performed with the latter cell line confirmed that Rab6 locates within the Golgi compartment (see Fig. 2C). In AR4-2J cells, Rab6 was associated with the cytosolic and microsomal fractions, but the protein was not detected in the cytosolic fraction of SOJ-6 cells. Regardless of the cell line or cell

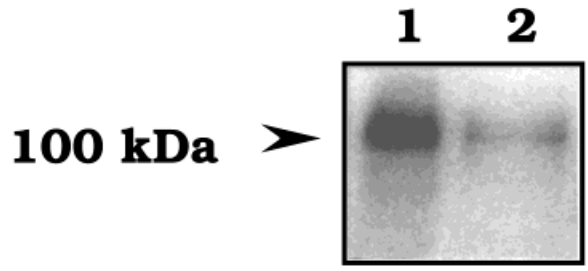

Fig. 3. Expression of BSDL in SOJ-6 cells. SOJ-6 cells were cultured for 6 h in fresh medium, the cell culture medium was removed. Cells were harvested, lysed, and fractionated by centrifugation into cytosolic and microsome fractions. Fifty μg protein from each fraction were separated on SDS-PAGE and electrotransferred onto nitrocellulose membrane. BSDL was then detected with *pAbL64* (1 μg/ml) and [^{125}I]-protein A (0.25 μCi/ml). The membrane was exhaustively washed, air-dried, and autoradiographed. Lane 1: microsome fraction; lane 2: cytosol fraction.

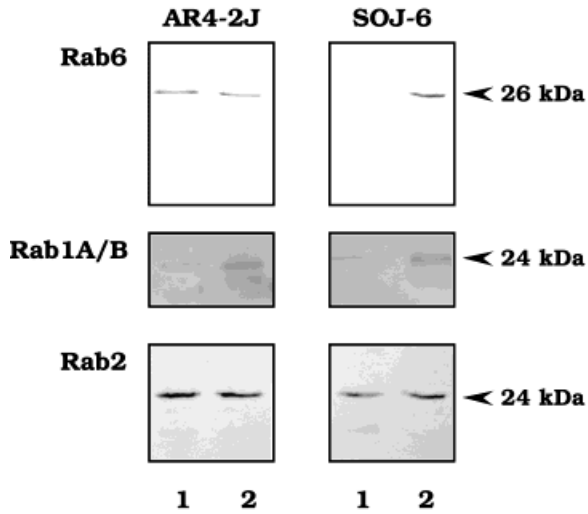

Fig. 4. Western-blotting analysis of various Rabp in soluble and membrane fractions of pancreatic cell lines. Protein (20–25 μg) from soluble and membrane fractions of AR4-2J and SOJ-6 cells was separated by SDS-PAGE and analyzed after electrotransfer to nitrocellulose membranes. Membranes were then probed with antibodies specific for Rab6, Rab1A/B, and Rab2. Detection was then performed with anti-rabbit alkaline phosphatase-conjugated or peroxidase-conjugated (chemiluminescence) antibodies. Lane 1: cytosol fraction; lane 2: microsome fraction.

fractions used, the immunoreactive signal can be suppressed by the competitive Rab6 peptide against which the antiserum was raised (not shown). The partitioning of Rab6 between cytosol and microsome membranes was also determined from overlay with [^{35}S]-αGTP and from Western blotting using [^{125}I]-protein A to detect the antigen-antibody complexes. Independent of the method used, data presented in Table II show that Rab6 partitions between cytosol and microsome membrane fractions of AR4-2J cells whereas in SOJ-6 cells, it was strictly associated with membranes, confirming data presented above. This study was extended to human normal and tumor pancreatic tissues using [^{35}S]-αGTP binding (Table II). Although Rab6 partitioned between cytosol and microsome membrane fractions equally in normal tissue, in tumoral tissue the protein appeared mainly associated with membranes. The partition of two other Rabp, Rab1A/B, and Rab2, was also examined by Western blotting using specific antibodies, data given on Figure 4 indicated that these two Rabp partitioned similarly in rat AR4-2J and human SOJ-6 cells. This result suggested that only the Rab6 partition could be affected in human tumoral pancreatic cells. Therefore, the cycle of Rab6 between donor and acceptor membrane could be inefficient or at least slower in SOJ-6 cells (and in human tumoral pancreatic tissue) than in AR4-2J cells (and in human normal tissue). Because Rab6 has been assumed to regulate vesicles transport in the Golgi apparatus, it is conceivable that a dysfunction of Rab6 may be at the origin of the defective BSDL secretion process observed in SOJ-6 cells and in human tumoral pancreas.

We next examined whether Rab6 was involved in the exocytosis process of pancreatic cells. Antisense DNA-oligonucleotides specific for Rab6 were used to prevent the secretion of BSDL. Subconfluent AR4-2J cells were incubated with the pair of ATG-antisense and inner-antisense oligonucleotides or with the reversed ATG-antisense described in Materials and Methods. During this incubation period, BSDL activity present in aliquots of the culture medium removed at time indicated was assayed (Fig. 5). When compared to control, the pair of antisense oligonucleotides decreased the rate at which AR4-2J cells secrete BSDL by 30–40%. This effect was significant after 70 h incubation. At this time, the amount of Rab6 is decreased by 42 (±5)% compared to control (Fig. 5, insert). The ability of antisense oligonucleotides to block secretion is not complete probably because of the long half-life of Rabp. Another reason is that the sequence of the ATG-antisense oligonucleotide which includes 5′ non-translated sequence was deduced from that of the human Rab6 cDNA [Zahraoui et al.,

TABLE II. Partitioning of Rab6 in Cell Lines and Human Pancreatic Tissue*

	Western-blotting		Immunoprecipitation	
	[^{125}I]-protein A overlay		[^{35}S]-αGTP overlay	
	Cytosol	Membranes	Cytosol	Membranes
AR4-2J cells	29.5	70.5	22.1	77.9
SOJ-6 cells	0	100	0	100
Human normal pancreatic tissue	n.d.	n.d.	43.6	56.5
Human tumoral pancreatic tissue	n.d.	n.d.	5.4	94.7

*Values are expressed as % of the total amount of rab6 detected by either method and are the average of two independent experiments. n.d.: not determined.

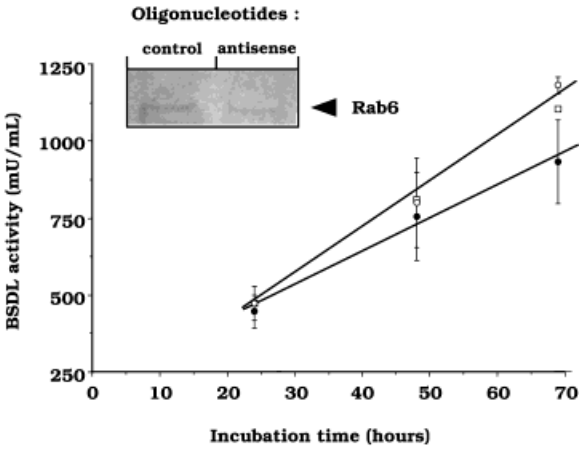

Fig. 5. Inhibition of BSDL secretion by antisense oligonucleotides. Subconfluent AR4-2J cells were incubated for 70 h in the presence of 5 μM of ATG–antisense and 5 μM inner-antisense oligonucleotides (●). As a control, cells were incubated with 10 μM of reversed–ATG–antisense oligonucleotides (○) or in the absence of oligonucleotides (□). Fresh oligonucleotides were added daily. At time as indicated, aliquots of the culture medium were withdrawn and the BSDL activity was measured using 4-nitrophenyl hexanoate. Data shown are the means ± standard deviation from three independent experiments. Insert shows a typical Western blotting performed on cell lysate (50 μg cell proteins by lane) after 70 h incubation with reversed-ATG-antisense oligonucleotide (control) or with ATG-antisense and inner-antisense oligonucleotides (antisense).

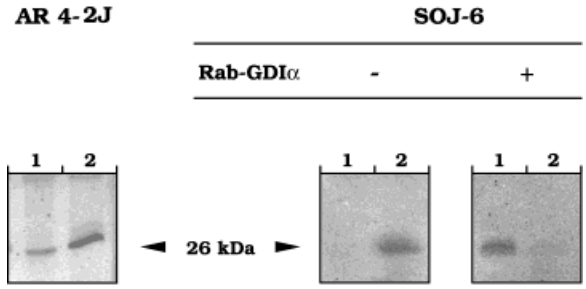

Fig. 6. Rab-GDI extraction and GTP-binding to Rab6. SOJ-6 cell lysate (200 μl) was pre-exchanged with 10 mM GDP (30°C, 10 min) and the exchange reaction was stopped by MgCl2 (40 mM). Cell lysate was then incubated (30°C, 40 min) with (+) or without (−) recombinant Rab-GDIα. At the conclusion of the incubation, microsome and cytosol fractions were separated by centrifugation. These fractions were also isolated from AR4-2J cells (without prior incubation with Rab-GDIα). The same amount of protein from soluble and microsome fractions were immunoprecipitated with 5 μg of antibodies specific for Rab6. Antigen-antibody complexes were separated on SDS-PAGE and electrotransferred onto nitrocellulose membranes. Blots were probed with [^{35}S]α-GTP (2 μCi/ml) and autoradiographed. Lane 1: cytosol fraction; lane 2: microsome fraction.

1989] and may differ from that of the corresponding rat mRNA. However, these data indicated that Rab6 is involved in the secretion process of BSDL by AR4-2J pancreatic cells.

Nucleotide Sequence of Rab6 Expressed by SOJ-6 Cells

We next attempted to determine the origin of dysfunctional Rab6 cycling in SOJ-6 cells. For this purpose, microsomal and cytosolic fractions of SOJ-6 and AR4-2J cells were immunoprecipitated using polyclonal antibodies specific for Rab6 and the immunoprecipitated material was separated by SDS-PAGE and transferred to nitrocellulose membranes. Rab6 present in the cytosolic and in microsome fractions of AR4-2J cells was able to bind [^{35}S]-αGTP in overlay experiments (Fig. 6, see also Table II). Binding of [^{35}S]-αGTP to Rab6 present in the microsome fraction of SOJ-6 cells was also detected but no binding was observed in the cytosolic fraction of these cells. This result suggests that Rab6 immunoprecipitated from the SOJ-6 cells microsome membrane fraction was functional and further confirms that the protein is not detectable within the cytosol fraction of these cells. Metabolic labeling of SOJ-6 cells with [^{14}C]-mevalonic

lactone in the presence of 10 μM lovastatin to block the endogenous isoprenoid synthesis [Wilson et al., 1996] followed by specific immunoprecipitation also indicated that Rab6 expressed in SOJ-6 cells can be geranylgeranylated (not shown).

Finally, to determine if any point mutations in Rab6 sequence were present that might induce the mislocalization of this GTP-binding protein expressed in human pancreatic tumoral SOJ-6 cells, total RNA was extracted, reverse transcribed and amplified by PCR using a pair of primers designed to amplify the entire sequence of the Rab6 cDNA. Under our conditions, one transcript was obtained from RNA of SOJ-6 and AR4-2J cells; its size was that expected (≈0.6 kb). The cDNA fragment obtained from SOJ-6 cells was subcloned into pCR2.1 vector and sequenced. Its sequence (GenBank AF 130986) is identical to that obtained from the human pheochromocytoma cells [Zahraoui et al., 1989]. [^{35}S]-αGTP binding, isoprenylation experiments and sequence analysis therefore indicates that Rab6 expressed in SOJ-6 cells is functional. Thus, defective cycling of this protein in human tumoral cells could originate from the dysfunction of a companion protein.

Expression of Rab-GDI by Pancreatic Cell Lines

Soluble Rab-GDIs continuously recycle Rabp from the acceptor back to the donor compartment, and play a pivotal role in shuttling of Rabp between the cytosol and membranes [Sasaki et al., 1990; Pfeffer et al., 1995]. Based on these results, we predicted that Rab6 could be released from SOJ-6 cell membranes by soluble proteins present in the cytosol of AR4-2J cells. To test this prediction, SOJ-6 cell proteins were radiolabeled with [^{35}S]-methionine and microsomes were isolated by centrifugation. Cytosol from unlabeled SOJ-6 and AR4-2J cells was also prepared. Radiolabeled membranes (150 μg of microsomal proteins) of SOJ-6 cells were added back to unlabeled cytosol (240 μg of soluble proteins) isolated from AR4-2J cells and then incubated for 30 min at 37°C. Control experiments were performed under identical conditions but using unlabeled SOJ-6 cytosol proteins. Cytosol and microsome membrane fractions were then collected by centrifugation and Rab6 was immunoprecipitated. After SDS-PAGE and Western blotting, the immunoreactive signal specific for Rab6 was quantitated by autoradiography. After correction, 20 (±4)% of labeled Rab6 was extracted from SOJ-6 microsome membranes by AR4-2J cell cytosol. Although the amount of released Rab6 seems low, this result suggests that the turn-over of Rab6 in SOJ-6 can be partly restored by soluble proteins from AR4-2J cells. To ascertain whether these proteins include Rab-GDIs, we used the Rab-GDI extraction assay described by Beranger et al. [1994]. SOJ-6 cell lysate was pre-exchanged with GDP (10 mM final concentration, 30°C, 10 min) and the exchange was stopped by adding MgCl2 (40 mM final). Cell lysates (200 μl) whether incubated with 2 μg recombinant Rab-GDI or with 2 μg BSA in Rab-GDI storage buffer (30°C, 40 min). At the end of incubation, microsome and cytosol fractions were isolated by ultracentrifugation. Finally, these fractions were immunoprecipitated with antibodies specific for Rab6, separated on SDS-PAGE and electrotransferred onto membranes followed by [^{35}S]–αGTP overlay. Under these conditions, Rab6 is mainly recovered in the cytosolic fraction (Fig. 6). This result strongly argues that a cytosolic protein, such as Rab-GDI is defective in SOJ-6 cells. We then explored this possible Rab-GDI defect. To this end, the presence of Rab-GDI in cytosolic and membrane fractions of both AR4-2J and SOJ-6 cells was investigated. As shown in Figure 7, antibodies specific for Rab-GDIα recognize a protein of approximately 57 kDa which is primarily associated with the cytosol of AR4-2J cells (arrowhead). Under our experimental conditions, Rab-GDIα

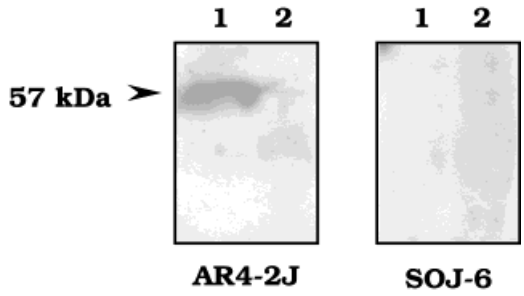

Fig. 7. Western-blotting analysis of Rab-GDI in cytosol and microsome fractions of pancreatic cells. Proteins (20 μg) of cytosol and microsome fractions of AR4-2J and SOJ-6 cells were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Blots were probed with antibodies specific for Rab-GDIα. The antigen-antibodies complex was detected using [^{125}I]-protein A. Lane 1: cytosol fraction; lane 2: microsome fraction.

cannot be detected in cytosol or membrane fractions of SOJ-6 cells. This difference could be due to the specificity (i.e., RabGDIα (or 1) could not be expressed in SOJ-6 cells) and/or to the reactivity of antibodies used. Therefore, we attempted to isolate RNA transcripts encoding Rab-GDI. For this purpose, RNA extracted from AR4-2J and SOJ-6 cells was reverse transcribed and the cDNA was amplified using specific primers to amplify rat and human Rab-GDIs. Following these RT-PCR, we were able to detect transcripts of approximately 1.4 kb encoding Rab-GDIα and β in rat cells and Rab-GDI1 and 2 in human cells (not shown). These results suggested that Rab-GDI proteins could be translated from their respective mRNA in rat and human pancreatic cells as well. We next attempted to determine whether these mRNAs can be translated into functional proteins. For practical reasons, we used a degenerated pair of primers common to rat and human Rab-GDI proteins and centered around sequences encoding Sequence-Concerved Region (SCR) such as SCR3B which is part of the putative Rabp binding and extraction domain [Schalk et al., 1996; Wu et al., 1998]. Under these conditions, a cDNA transcript of the expected size (approximately 1 kb) was obtained in AR4-2J cells. This cDNA fragment was cloned into pCR2.1 vector and sequenced (GenBank, AF 130987). Its sequence was 75.8 and 100% homologous to that of Rab-GDIβ and Rab-GDIα, respectively [Nishimura et al., 1994]. Once translated into protein, the sequence of the AR4-2J cells transcript was identical to the α- or 1-isoform of rat Rab-GDI proteins (Fig. 8). When RNA isolated from SOJ-6 cells was used to program the RT-PCR experiment, transcripts of approximately 1 kb were also amplified and cloned as above. Strikingly, the sequence of these fragments was identical (two clones obtained from independent RT-PCR experiments were sequenced) and had some 73.7% and 99.6% sequence homologies with the α- and β-isoforms of human Rab-GDI, respectively. When these common degenerated primers were used to amplify RNA extracted from normal human pancreas, a cDNA fragment of 1 kb was also isolated. The sequence of this transcript (GenBank AF 144713) is identical to that of the β-isoform of Rab-GDI. When compared, sequences of transcripts amplified from SOJ-6 cells and human pancreas RNA showed differences and point mutations were observed at nucleotide positions 355, 531, 671, and 737. Examining amino acid sequences deduced from the SOJ-6 cell, Rab-GDIβ cDNA fragment indicated that it was 97.8% homologous to the human Rab-GDIβ (Fig. 8). Although mRNA specific for the α (or 1) and β isoforms of Rab-GDI were amplified in AR4-2J and SOJ-6 cells, these results suggested that the β-isoform seems preferentially amplified by common primers in human pancreas and in human tumoral cells whereas the α-isoform was amplified by these primers in AR4-2J cells. This also suggests that Rab-GDIβ could be preferentially expressed in human pancreas. This agrees not only with the lack of Rab-GDIα antibodies reactivity in SOJ-6 cells (see Fig. 7) but also with the fact that recombinant Rab-GDIα is able to extract Rab6 from microsome membranes of SOJ-6 cells, supportive that if Rab-GDIα was expressed no impairment of Rab6 cycling would occur in the latter cells. This, *a contrario*, indicates that Rab-GDIα could not be the main Rab-GDI expressed in SOJ-6 cells. In the same way rat pancreatic AR4-2J cells seem to preferentially express the α-isoform. Alignment with known Rab-GDI sequences (Fig. 8) indicated that each nucleotide mutation of the Rab-GDIβ fragment isolated from SOJ-6 cells results in amino acid substitutions; two of them are conservative (L304V and V326A) whereas the other two are not (E257V and D199H). Amino acids essential for the Rab-GDI function await to be clearly defined. Nevertheless, the recent determination of the brain Rab-GDIα isoform crystal structure [Schalk et al., 1996; Wu et al., 1998] lead to the definition of Sequence-Conserved Regions (or SCRs) which contribute to the Rab-GDI function (see Fig. 8). Despite being separated by nearly 140 residues SCR 3B winds back to contact SCR 1 and form the apex domain I which represents the putative Rab binding region. Amino acid substitutions in the SCR 3B domain abolish Rabp/Rab-GDI complex formation, inhibit Rabp extraction from membranes and impaired vesicular transport in vitro. The other two Sequence-Conserved Regions, SCR 2 and SCR 3A, represent candidate regions for interaction with receptors mediated Rabp binding to membranes. One of the non-conservative substitutions in SOJ-6 cells Rab-GDIβ, E257V, was located within the SCR 3B, whereas the second one, D199H, was proximal to the SCR 3A. The

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GDIα-Bovine      MDEEYDVIVLGTGLTECILSGIMSVNGKKVLHMDRNPYYGGESSSITPLEELYKRFQLLE   60
GDI1-Mouse       ------------------------------------------------------------
GDI1-Rat         MDEEYDVIVLGTGLTECILSGIMSVNGKKVLHMDRNPYYGGESSSITPLEELYKRFQLLE
GDIα-Human       MDEEYDVIVLGTGLTECILSGIMSVNGKKVLHMDRNPYYGGESSSITPLEELYKRFQLLE
GDIα-AR42J       ------------------------------------------------------------
GDI2-Mouse       MNEEYDVIVLGHWLTECILSGIMSVNGKKVLHMDRNPYYGGESASITPLEDLYKRFKIPG
GDI2-Rat         MNEEYDVIVLGTGLTECILSGIMSVNGKKVLHMDQNPYYGGESASITPLEDLYKRFKLPG
GDIβ-Human       MDEEYDVIVLGTGLTECILSGIMSVNGKKVLHMDRNPYYGGESASITPLEDLYKRFKIPG
GDIβ-SOJ6        ------------------------------------------------------------
                          SCR 1A          SCR 1B

GDIα-Bovine      GPPETMGRGRDWNVDLIPKFLMANGQLVKMLLYTEVTRYLDFKVVEGSFVYKGGKIYKVP   120
GDI1-Mouse       ------------------------------------------------------------
GDI1-Rat         GPPESMGRGRDWNVDLIPKFLMANGQLVKMLLYTEVTRYLDFKVVEGSFVYKGGKIYKVP
GDIα-Human       GPPESMGRGRDWNVDLIPKFLMANGQLVKMLLYTEVTRYLDFKVVEGSFVYKGGKIYKVP
GDIα-AR42J       --------------------LMANGQLVKMLLYTEVTRYLDFKVVEGSFVYKGGKIYKVP
GDI2-Mouse       APPASMGRGRDWNVDLIPKFLMANGQLVKMLLFTEVTRYLDFKVTEGSFVYKGGKIYKVP
GDI2-Rat         QPPASMGRGRDWNVDLIPKFLMANGQLVKMLLFTEVTRYMDFKVIEGSFVYKGGKIYKVP
GDIβ-Human       SPPESMGRGRDWNVDLIPKFLMANGQLVKMLLYTEVTRYLDFKVTEGSFVYKGGKIYKVP
GDIβ-SOJ6        --------------------LMANGQLVKMLLYTEVTRYLDFKVTEGSFVYKGGKIYKVP
                                     ************:******:**** ***************
                                                SCR 2

GDIα-Bovine      STETEALASNLMGMFEKRRFRKFLVFVANFDENDPKTFEGVDPQNTSMRDVYRKFDLGQD   180
GDI1-Mouse       ----EALRSNLMGMFEKRRFRKFLVFVANFDENDPKTFEGVDPQNTSMRDVYRKFDLGQD
GDI1-Rat         STETEALASNLMGMFEKRRFRKFLVFVANFDENDPKTFEGVDPQTTSMRDVYRKFDLGQD
GDIα-Human       STETEALASNLMGMFEKRRFRKFLVFVANFDENDPKTFEGVDPQTTSMRDVYRKFDLGQD
GDIα-AR42J       STETEALASNLMGMFEKRRFRKFLVFVANFDENDPKTFEGVDPQTTSMRDVYRKFDLGQD
GDI2-Mouse       STEAEALASSLMGLFEKRRFRKFLVYVANFDENDPRTFEGIDPKKTSMREVYKKFDLGQD
GDI2-Rat         STEAEALASSLMGLFEKRRFRKFLVYVANFDEKDPRTFEGVDPKKTSMRDVYKKFDLGQD
GDIβ-Human       STEAEALASSLMGLFEKRRFRKFLVYVANFDEKDPRTFEGIDPKKTTMRDVYKKFDLGQD
GDIβ-SOJ6        STEAEALASSLMGLFEKRRFRKFLVYVANFDEKDPRTFEGIDPKKTTMRDVYKKFDLGQD
                     *** *.***:***********:******:**:****:**:.*:**:**:*******
                              SCR 2 (continued)

GDIα-Bovine      VIDFTGHALALYRTDDYLDQPCLETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR   240
GDI1-Mouse       VIDFTGHALALYRTDEYLDQPCLETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
GDI1-Rat         VIDFTGHALALYRTDDYLDQPCLETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
GDIα-Human       VIDFTGHALALYRTDDYLDQPCLETVNRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
GDIα-AR42J       VIDFTGHALALYRTDDYLDQPCLETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
GDI2-Mouse       VIDFTGHALALYRTDDYLDQPCCETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
GDI2-Rat         VIDFTGHSLALYRTDDYLDQPCCERLTELNFYSESLARYGKSPYLYPLYGPWELPQGFAR
GDIβ-Human       VIDFTGHALALYRTDDYLDQPCYETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
GDIβ-SOJ6        VIDFTGHALALYRTDDYLHQPCYETINRIKLYSESLARYGKSPYLYPLYGLGELPQGFAR
                 *******:*******:**.*** * :..:::********************  ********
                                                SCR 3A
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Fig. 8. Comparison of the amino acid sequence of Rab-GDI transcript with Rab-GDIs sequences. Accession numbers for the sequences used are rat Rab-GDIα (GenBank X74402), rat Rab-GDIβ (GenBank X74401), human Rab-GDIα (GenBank D45021), human Rab-GDIβ (GenBank D13988), bovine Rab-GDI1 (GenBank D90103), mouse Rab-GDI1 (GenBank U07950), and mouse Rab-GDI2 (GenBank U07951). Stars indicated matches; dots, conserved amino acids, and absence of label indicates mismatches. Amino acid substitutions detected in the SOJ-6 Rab-GDI transcript are underlined and in bold letters. Double underlined sequences are representative of Sequence-Conserved Regions (SCR) of Rab-GDI as defined by Schalk et al. [1996].

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GDIα-Bovine     LSAIYGGTYMLNKPVDDIIMENGKVVGVKSEGEVARCKQLICDPSYVPDRVRKAGQVIRI   300
GDI1-Mouse      LSAIYGGTYMLNKPVDDIIMENGKVVGVKSEGEVARCKQLICDPSYIPDRVQKAGQVIRI
GDI1-Rat        LSAIYGGTYMLNKPVDDIIMENGKVVGVKSEGEVARCKQLICDPSYIPDRVRKAGQVIRI
GDIα-Human      LSAIYGGTYMLNKPVDDIIMENGKVVGVKSEGEVARCKQLICDPSYIPDRVRKAGQVIRI
GDIα-AR42J      LSAIYGGTYMLNKPVDDIIMENGKVVGVKSEGEVARCKQLICDPSYIPDRVRKAGQVIRI
GDI2-Mouse      LSAIYGGTYMLNKPIEEIIMQNGKVIGVKSEGEIARCKQLICDPSYVKDRVEKVGQVIRV
GDI2-Rat        LSAIYGGTYMLNKPIEEIIVQNGKVVGVKSEGEIARCKQLICDPSYVKDRVEKVGQVIRV
GDIβ-Human      LSAIYGGTYMLNKPIEEIIVQNGKVIGVKSEGEIARCKQLICDPSYVKDRVEKVGQVIRV
GDIβ-SOJ6       LSAIYGGTYMLNKPIEVIIVQNGKVIGVKSEGEIARCKQLICDPSYVKDRVEKVGQVIRV
                ***************:: **::****:*******:************: ***.*.*****:

                     SCR 3B

GDIα-Bovine     ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISYAHNVAAQGKYIAIASTTVETT   360
GDI1-Mouse      ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISYAHNVAAQGKYIAIASTTVETA
GDI1-Rat        ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISYAHNVAAQGKYIAIASTTVETA
GDIα-Human      ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISYAHNVAAQGKYIAIASTTVETT
GDIα-AR42J      ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISYAHNVAAQGKYIAIASTTVETA
GDI2-Mouse      ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISSAHNVAAQGKYIAIASTTVETK
GDI2-Rat        ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISFAHNVAAQGKYIAIVSTTVETK
GDIβ-Human      ICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISFAHNVAAQGKYIAIVSTTVETK
GDIβ-SOJ6       ICIVSHPIKNTNDANSCQIIIPQNQANRKSDIYVCMISFAHNVAAQGKYIAIVSTTVETK
                ***:********************.************ *************.******

GDIα-Bovine     DPEKEVEPALELLEPIDQKFVAISDLYEPIDDGSESQVFCSCSYDATTHFETTCNDIKDI   420
GDI1-Mouse      EPEKEVEPALELLEPIDQKFVAISDLYEPIDDGSESQVFCSCSYDATTHFETTCNDIKDI
GDI1-Rat        EPEKEVEPALELLEPIDQKFVAISDLYEPIDDGSESQVFCSCSYDATTHFETTCNDIKDI
GDIα-Human      DPEKEVEPALELLEPIDQKFVAISDLYEPIDDGCESQVFCSCSYDATTHFETTCNDIKDI
GDIα-AR42J      EPEKEVEPALELLEPIDQKFVAISDLYEPIDDGSESQVFCSCSYDATTHFETTCNDIKDI
GDI2-Mouse      EPEKEIRPALELLEPIEQKFVSISDLLVPKDLGTESQIFISRTYDATTHFETTCDDIKDI
GDI2-Rat        EPEKEIRPALELLEPIEQKFVSISDLFVPKDLGTDSQIFISRAYDATTHFETTCDDIKDI
GDIβ-Human      EPEKEIRPALELLEPIEQKFVSISDLLVPKDLGTESQIFISRTYDATTHFETTCDDIKNI
GDIβ-SOJ6       EPEKEIRPALELLEPIEQKFVSISDLLVPKDLGTESQIFISRTYDATTHFETTCDDIKNI
                :****:.*********:****:****  * * * :**:* * :***********:***:*

GDIα-Bovine     YKRMAGSAFDFENMKRKQNDVFGEADQ                                   427
GDI1-Mouse      YKRMAGSAFDFENMKRKQNDVFGEADQ
GDI1-Rat        YKRMAGSAFDFENMKRKQNDVFGEADQ
GDIα-Human      YKRMAGTAFDFENMKRKQNDVFGEAEQ
GDIα-AR42J      YKRMAGSAFDFENMKRKQN--------
GDI2-Mouse      YKRMMGSEFDFEEMKRKKNDIYGEE--
GDI2-Rat        YKRMTGSEFDFEEMKRKKNDIYGED--
GDIβ-Human      YKRMTGSEFDFEEMKRKKNDIYGED--
GDIβ-SOJ6       YKRMTGSEFDFEEMKRKKN--------
                **** *: ****:******
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Figure 8. (Continued.)

nature and location of these two substitutions are predicted to result in a loss of Rab-GDIβ function [Schalk et al., 1996; Wu et al., 1998] and suggest that the mutated Rab-GDIβ expressed by human tumoral SOJ-6 cells could be unable to extract Rab6 from subcellular membranes.

Transport of Transmembrane Glycoproteins

We next investigated whether the defect of Rab6 cycling in SOJ-6 cells could affect the trafficking of other glycoproteins and their delivery to membranes. For this purpose, we analyzed the presence of two membrane proteins in SOJ-6 cells: the transferrin receptor (or

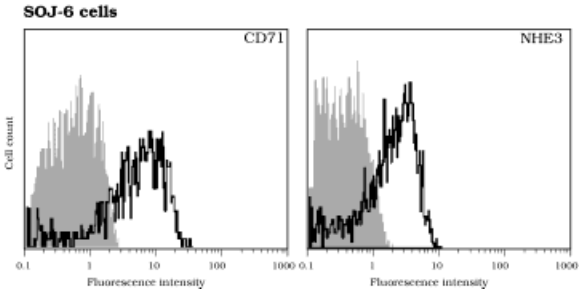

Fig. 9. Flow cytometric analysis of CD71 and NHE3 cell surface antigens. SOJ-6 cells were grown to 80% confluence and dissociated with non-enzymatic Cell Dissociation Solution before incubation with appropriate antibodies and cytometric analysis. For each panel, the dark histogram shows the non-specific binding of FITC-labeled secondary antibodies and the open histogram illustrates data obtained with specific antibodies to CD71 (clone My29) and NHE3.

CD71), which is glycosylated [Hentze et al., 1996] and the sodium/proton exchanger (NHE3) which is not [Tse et al., 1992]. These two membrane proteins were first detected by Western blotting performed on AR4-2J and SOJ-6 cell lysates (not shown). Flow cytometry analysis confirmed that both NHE3 and CD71 were expressed and correctly localized in SOJ-6 cells (Fig. 9). Immunofluorescence microscopy of permeabilized SOJ-6 cells confirmed the expression of CD71 receptor by these cells (Fig. 10A). Punctate labeling appeared within the cytoplasm, consistent with the endosomal location of this protein. When SOJ-6 cells were not permeabilized prior to the immunofluorescence experiment, CD71 labeling appeared restricted to the cell surface (Fig. 10B). The presence of these proteins at the cell surface indicate that they are correctly targeted to the plasma membrane of human pancreatic tumor cells. Consequently, expression of the defective Rab-GDIβ isoform in SOJ-6 cells appears to primarily affect the secretion of pancreatic enzymes such as BSDL but it does not influence the intracellular transport of membrane-associated proteins to their final destination.

DISCUSSION

The proteins of the Rab family are major elements of vesicular transport within eukaryotic cells. Rabp have been localized to various compartments of both secretory and endocytic pathways. These small GTPases are involved in docking and fusion mechanisms of transport vesicles with their membrane targets. Extensive studies performed on Rab6 demonstrate that it cycles between GDP-bound cytosolic and GTP-bound membrane forms [Novick and Zerial, 1997]. The alteration of Rab function profoundly disturbs the morphology of organelles, consistent with its essential role in vesicle targeting. For exemple, overexpression of the GTP-bound form of Rab1A, a protein involved in the ER to Golgi transport [Tisdale et al., 1992], affects the morphology of the Golgi apparatus [Wilson et al., 1994]. Similarly, mutants of Rab6 redistribute Golgi resident proteins into the ER [Martinez et al., 1997].

A model of the Rabp functional mechanism has been proposed [Novick and Zerial, 1997; Pfeffer, 1994] in the cytosol, Rabp are found as complexes with a Rab-GDP dissociation inhibitor (GDI) [Pfeffer et al., 1995]. This Rab-GDI protein could deliver the Rabp to a specific organelle [Soldati et al., 1994]. The association of the Rabp with membranes and its subsequent guanine nucleotide exchange is then catalyzed by a Rab-GDI displacement factor (GDF) and a guanine nucleotide exchange factor (GEF), respectively [Novick and Zerial, 1997; Pfeffer, 1994]. The GTP-bound Rabp is thus recruited onto donor transport vesicles to interact with one or more downstream effectors. The ultimate role of Rabp and partners such as Rab-GDI may be to provide fidelity in the process of docking and/or fusion of vesicles with the proper acceptor compartment [Rothman, 1994]. Following fusion, the hydrolysis of GTP by the intrinsic GTPase activity of Rabp, possibly stimulated by a GTPase-activating protein (GAP), would convert GTP-bound into GDP-bound Rabp. This GDP-bound Rabp could then be recycled to the cytosol through the action of Rab-GDI protein that is able to extract the GDP-bound Rabp from the acceptor membrane [Soldati et al., 1994; Rothman, 1994; Ullrich et al., 1994]. The phosphorylation of the cytosolic Rab-GDI/Rabp complex may then allow Rab-GDI to enter a new cycle. In this context, Rab-GDI has a pivotal role as an important element that regulates the functional cycle of Rabp (e.g., in a dynamic equilibrum between the cytosol and the subcellular membrane).

There are thought to be different mechanisms within the ER for the proper segregation of cargo molecules into vesicles. One of them is the so-called quality control system, which is a

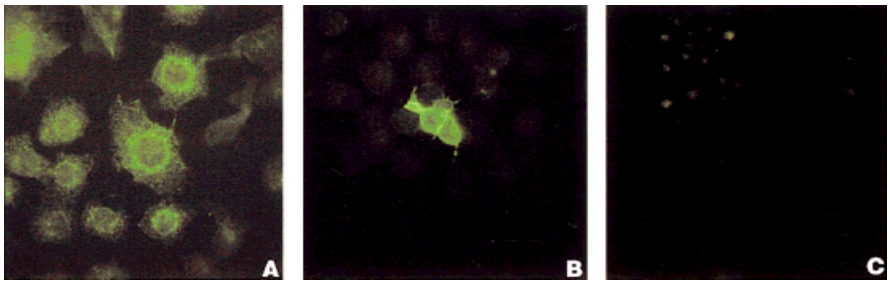

Fig. 10. Expression and localization of CD71 antigen in SOJ-6 cells. SOJ-6 cells were permeabilized with 0.05% saponin in PBS buffer and incubated with antibodies specific to CD71 (clone My29) and FITC-labeled antibodies directed against IgG (**A**). The same experiment was performed on SOJ-6 cells without prior permeabilization of cell membrane (**B**). For control experiments, SOJ-6 cells were incubated with the FITC-labeled secondary antibodies and omitting primary antibodies (**C**).

retention process whereby incompletely folded proteins are kept from entering cargo vesicles because they remain bound to ER resident proteins such as those of the chaperone family. This mechanism would explain the accumulation of BSDL within the ER-Golgi compartment and the correct targeting of membrane (glyco) proteins such as CD71 and NHE3. We have shown that a membrane multiprotein complex including the chaperone Grp94 assists in the folding and the secretion of BSDL [Bruneau and Lombardo, 1995]. This association is required for the complete glycosylation of the enzyme [Bruneau et al., 1997]. However, once fully glycosylated [Abouakil et al., 1993; Bruneau et al., 1997], the protein is secreted and thus the structure of glycans does not interfere with the secretion process of BSDL [Abouakil et al., 1993; Mas et al., 1997; Bruneau et al., 1997]. Although, it is possible that the protein itself is mutated in the SOJ-6 cells, leading to its missorting from the *trans*-Golgi compartment, we have recently cloned the BSDL variant expressed in SOJ-6 cells [Pasqualini et al., 1998]. The sequence of the SOJ-6 cDNA variant of BSDL is identical to that of BSDL expressed by the human normal pancreas, however part of the mucin-like C-terminal domain was deleted leading to the presence of six, instead of 16, tandemly repeated sequences [Pasqualini et al., 1998]. Nevertheless, this deletion does not affect the secretion of this variant which is observed after transfection of CHO cells. A protein with molecular properties identical to the BSDL variant expressed by SOJ-6 cells and termed feto-acinar pancreatic protein [Escribano and Imperial, 1989] or Concanavalin-A reactive variant [Mas et al., 1997], has been detected in the human pancreatic secretion where it accounts for some 30% of the BSDL mass [Mas et al., 1997; Pasqualini et al., 1998]. Consequently, the retention of BSDL in SOJ-6 cells does not appears to result from inherent properties or mis-folding of the protein, but rather, a defective vesicular transport may explain the accumulation of BSDL in SOJ-6 cells.

The exact nature of the compartment where BSDL accumulates is rather unprecise. Localization by confocal microscopy suggests that the cellular retention compartment may be the ER [Miralles et al., 1993]. However, BSDL expressed by SOJ-6 cells carries out the oncofetal *J28* epitope which requires glycosyltransferases active in the Golgi [Panicot et al., 1999; Mas et al., 1997]. Furthermore, BSDL should also reach the *trans*-Golgi compartment where fucosyltransferases locate [Colley, 1997] to acquire this epitope. Subcellular fractionation indicates that BSDL expressed by human pancreatic tumor cells is associated with a compartment, most likely the Golgi apparatus, which displays the highest incorporation of galactose into proteins [Roudani et al., 1994]. In SOJ-6 cells, BSDL co-locates with the 58K-Golgi protein. However, subcellular fractionation and immunofluorescence studies also detect BSDL in the ER. These results may be reconciled by the consideration that a retrograde transport from the Golgi to the ER occurs; Rab6 that controls intra-Golgi retrograde transport [Martinez and Goud, 1998; White et al., 2000] may also control this transport [Martinez et al., 1994]. Indeed, it has been recently shown that membrane-associated GTP-bound mutants of Rab6 induce a brefeldin A-like ef-

fect and redistribute Golgi resident proteins into the ER [Martinez et al., 1994]. Consistent with this observation, is the strict association of Rab6 with SOJ-6 microsome membranes whereas it partitions between the cytosol and membranes of AR4-2J cells. When Rabp accumulate at their membrane targets, subsequent transport events cannot take place. Therefore, dysfunctioned cycling of Rab6 may explain fucosylation in the Golgi [Panicot et al., 1999; Mas et al., 1997] and the ER retention of BSDL [Miralles et al., 1993]. Rab6 in both SOJ-6 and AR4-2J cells is able to bind [^{35}S]-αGTP and consequently appears functional. Furthermore, the sequence of Rab6 cloned from SOJ-6 cells indicates that the protein is intact and normal. To bind to membranes, Rabp must be prenylated [Casey and Seabra, 1996; Shen and Seabra, 1996]. Because in SOJ-6 cells, Rab6 is found associated with the membrane, one may assume that the protein is prenylated as actually shown here. This finding eliminates the notion that geranylgeranyl-transferase(s) or Rab escort protein(s) (REP) are not functional. Taken together, all these data suggested that Rab6 expressed by SOJ-6 cells should normally cycle between the cytosol and the membrane fractions of these cells. Obviously, the absence of such cycling and the retention of Rab6 to membranes could arise from a defective Rab6 companion protein. Albeit mRNA encoding each Rab-GDI isoform was evidenced by RT-PCR in the two cells lines tested, our study suggests that a Rab-GDIα isoform [Nishimura et al., 1994; Fukui et al., 1997] and the β-isoform are preferentially expressed in rat AR4-2J and in human SOJ-6 cells, respectively. Moreover, the partial amino acid sequence of Rab-GDIβ expressed in SOJ-6 cells displays a few amino acid substitutions when compared to native Rab-GDIs sequences. Among these mutated residues, one located in position 257 (E257V) forms part of the sequence-concerved SCR 3B region which is likely involved in Rabp binding and extraction [Schalk et al., 1996; Wu et al., 1998]. A second one, D199H, is located proximal to SCR 3A which represents a region for interaction with receptors mediating Rabp binding to membranes [Schalk et al., 1996]. These two substitutions which arise in essential domains of Rab-GDIs could explain the failure of Rab-GDIβ function in cycling Rab6 in SOJ-6 cells. This possibility is substantiated by the demonstrated ability of the recombinant Rab-GDIα to efficiently extract in vitro Rab6 from SOJ-6 cells subcellular membranes. However, it is possible that the β-isoform and α-isoform of Rab-GDI, could be involved, under physiological conditions, in the cycling of different Rabp even if in vitro each Rab-GDI isoform is able to extract any Rabp [Janoueix-Lerosey et al., 1995]. This hypothesis is substantiated by the similar partition of Rab1A/B and Rab2 in each cell lines used in this study. Interestingly, it has been proposed that these latter Rabp are involved in the anteretrograde vesicular transport whereas Rab6 could be involved in the retrograde motion of cargo vesicles [Martinez and Goud, 1998]. Whether different Rab-GDI, depending upon the species examined, are involved in retrograde and anteretrograde transport has to be defined. A straightful way to demonstrate that mutated Rab-GDIβ is responsible for the defect in BSDL secretion by human pancreatic SOJ-6 cells would be to transfect these cells with the full length cDNA encoding the functional Rab-GDIα cloned from rat pancreatic AR4-2J cells. Albeit many transfection attempts, we were unable to transfer any cDNA within SOJ-6 cells. This ineffectiveness in transfecting SOJ-6 cells independently of the cDNA (E. Mas, personal communication) could likely be due to the high amount of mucoids present at the surface of SOJ-6 tumoral cells.

The ability of Rab-GDI to retrieve Rabp from membranes and to deliver them to their cognate membrane targets indicates that expression of a defective Rab-GDIβ in SOJ-6 cells would interfere with a variety of intracellular transport events [Garrett et al., 1994]. However, we show that membrane (glyco) proteins CD71 and NHE3 were normally targeted to the plasma membrane. These findings suggest that pancreatic secretory proteins and membrane proteins could be delivered to their appropriate destination by different routes. The presence of these two putative sorting pathways to the pancreatic cell surface is compatible with the function of this cell. The surface of all epithelial cells is typically divided into at least two functionally and biochemically distinct, but physically continuous domains. The apical domain faces the organ lumen, where pancreatic cells release soluble secretory proteins during exocytosis. The other domain is the basolateral cell surface, which harbors most of the plasma

membrane proteins required for the fundamental cellular processes such as the transferrin receptor CD71. Apical- and basolateral-directed membrane proteins are synthesized in the ER and together are transported to and through a common Golgi complex, but are finally segregated in the TGN and presented to the cognate membrane domain. Tumor pancreatic cells, even if they have lost their original polarity, should still exhibit some degree of asymmetry [Matter and Mellman, 1994]. This is not surprising because distinct apical- and basolateral-destined pathways are not a unique property of polarized cells since non-polarized fibroblasts also appear to have two trafficking routes from the TGN to the plasma membrane termed apical- and baso-lateral pathway [Yoshimori et al., 1996]. The importance of each of the two routes, however, depends on the cell type and on the protein in question [Matter and Mellman, 1994]. It is also known that in hepatocytes, membrane and secretory proteins appear to move to the cell surface in separate vesicle populations [Saucan and Palade, 1994]. In other cell type, such as fibroblasts, membrane fusion in the related apical pathway appears independent of N-ethylmaleimide-sensitive factor (NSF) activity and is not inhibited by Rab-GDI, as seen in polarized epithelia [Ikonen et al., 1995]. Therefore one may postulate that in pancreatic cells, membrane receptors CD71 and NHE3 follow the default itinerary independent of Rabp cycling [Ikonen et al., 1995] whereas BSDL is associated with those vesicles of the TGN, which requires selected proteins including Rab-GDI.

In conclusion, we theorize that Rab6 could be involved in the secretion processes of acinar pancreatic cells. While Rab6 cycles normally between the cytosol and membranes in secreting AR4-2J cells, this cycle seems to be impaired in non-secreting human pancreatic cancer cells (SOJ-6 cells). We propose that this deficiency is due to the expression of defective Rab-GDIβ protein by the latter cells. It remains to be shown whether the expression of mutated Rab-GDIβ is a specific factor among the pancreatic oncogenese or if it can be extended to all tumor secretory epithelial cells. The vast majority of pancreatic tumors (83%) are found to have accumulated alterations leading to *K*-ras oncogene activation and *p16* and *p53* tumor suppressor genes inactivation [Rozenblum et al., 1997]. Our findings underscore the multigenic nature of pancreatic cancers. Perhaps the unique expression of BSDL (or FAPP) in pancreatic tumor cells and the inactivation of their secretory pathway provide these cells with a strong selective proliferating advantage and help to supply cholesteryl esters for membrane biogenesis [Le Petit-Thévenin et al., 1998]. Although it is speculative to extrapolate data obtained with model cell lines to human pathology, the difference observed in the partitioning of Rab6 in normal and tumoral human pancreatic tissue (see Table II) strongly suggests that the impairments of the secretion process seen in pancreatic cancer could also be due to a defect of Rab6 cycling. Therefore, this study may represent a new case of deficiency of the vesicular transport in human pathology [Seabra, 1996].

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