Subcellular Distribution and Targeting of the Intracellular Chloride Channel p64

Christopher Redhead,* Stephen K. Sullivan,^{†‡} Chizuko Koseki,[§]
Keigi Fujiwara,[§] and John C. Edwards^{||¶}

*Max Planck Institut für Zuchtungsforschung, Köln, Germany; [†]Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032; [§]National Cardiovascular Centre, Fugishirodai, Osaka, Japan; and ^{||}Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

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p64 is an intracellular chloride channel originally identified in bovine kidney microsome. Using a combination of immunofluorescent and electron microscopic techniques, we demonstrate that p64 resides in the limiting membranes of perinuclear dense core vesicles which appear to be regulated secretory vesicles. Heterologous expression of p64 in Panc1 cells, a cell type which does not normally express p64, results in targeting to a similar compartment. Mutagenesis experiments demonstrate that both the N- and C-terminal domains of the protein independently contribute to subcellular distribution of the protein. The C-terminal domain functions to prevent expression of p64 on the plasma membrane and the N-terminal domain is necessary to deliver p64 to the appropriate membrane compartment.

INTRODUCTION

Eukaryotic cells contain intracellular membrane-bound organelles which, like the cell itself, control their internal milieu by a combination of active and passive transport mechanisms in their limiting membranes. Chloride channels are known to be present in membranes of several organelles and the regulation of these channels has been implicated in the control of a variety of cellular functions. The roles of chloride channels in intracellular physiology has been most clearly demonstrated in two processes: 1) acidification of intracellular compartments and 2) exocytosis of certain secretory vesicles. In both of these instances, the chloride conductance functions to short circuit an electrogenic cation transport mechanism.

The pH of intracellular organelles is tightly regulated and is critical to a number of organellar activities (Mellman *et al.*, 1986) including ligand-receptor interactions (e.g., see Dautry-Varsat, 1986), sialylation re-

actions (Barasch *et al.*, 1991), loading of certain secretory vesicles (Wolosker *et al.*, 1996; Bauerfeind and Huttner, 1993), and processing of prohormones (Orci *et al.*, 1987). Organellar acidification is carried out by the parallel actions of an electrogenic proton ATPase and a chloride conductance. The ATPase provides the proton-motive force necessary to generate a pH gradient while the chloride conductance short circuits the membrane potential generated by the pump and thus allows mass proton transport (Al-Awqati, 1986; Van Dyke, 1988). The chloride conductance not only permits acidification but also may regulate it. In clathrin-coated vesicles (Mulberg *et al.*, 1991), kidney endosomes (Bae and Verkman, 1990), and secretory granules of thyroid parafollicular cells (Barasch *et al.*, 1988; Tamir *et al.*, 1994), acidification appears to be actively regulated by modulation of the chloride conductance.

Chloride channels have also been implicated in the exocytosis of certain secretory vesicles independent of acidification. Exocytosis of salivary and pancreatic secretory granules is dependent on cytoplasmic anions and may be blocked by chloride channel inhibitors (Pazoles and Pollard, 1978; Dayanithi and Nordmann, 1989; Marcon *et al.*, 1990; Takuma and Ichida, 1991). In

[‡] Present address: Laboratory of Cardiac/Membrane Physiology, The Rockefeller University, New York, NY 10021.

[¶] Corresponding author: Renal Division, Barnes-Jewish Hospital North Campus, 216 South Kingshighway, St. Louis, MO 63110.

salivary and exocrine pancreatic secretory granules, secretagogues cause activation of chloride channels in the granule membrane prior to exocytosis (Pazoles *et al.*, 1980; Gasser *et al.*, 1988; Fuller *et al.*, 1989a,b). It is thought that concerted activation of these chloride channels and ATP-sensitive potassium channels in the secretory vesicle membrane allows influx of potassium chloride, leading to osmotic swelling of the granule prior to exocytosis.

If chloride conductance independently regulates acidification in various distinct but closely opposed membrane compartments as well as regulates exocytosis of certain secretory granules, it follows that chloride channels with distinct regulatory properties must be accurately targeted to the appropriate membranes. Mechanisms responsible for the subcellular distribution of membrane proteins have been the subject of intense investigation. Targeting takes place by a variety of mechanisms including retention/retrieval mechanisms, as along the exocytic pathway (Nilsson and Warren, 1994), direct delivery to the appropriate subcellular domain, as with mitochondrial proteins (Hohfeld and Hartl, 1994), and active sorting to compartment-destined vesicles in the trans-Golgi network, as with some polarized plasma membrane proteins of epithelia (Wandinger-Ness *et al.*, 1990; Matter and Mellman, 1994). In each of these instances, targeting depends on interaction of short amino acid sequence motifs (usually within portions of the protein exposed on the cytoplasmic face of the membrane) with other cellular structures.

Recently, p64, a chloride channel protein from bovine kidney microsomal membranes, has been purified and cloned (Landry *et al.*, 1989, 1993). Identification of p64 as a chloride channel protein rests on, first, the copurification of p64 with channel activity and, second, that immunodepletion of p64 from solubilized microsomal membranes with affinity-purified anti-p64 antibodies removes the reconstitutable chloride channel activity (Redhead *et al.*, 1992).

In this study, we show that the p64 is specifically located in the membranes of what appear to be regulated secretory vesicles. We characterize structural features of p64 which lead to proper subcellular distribution of the protein and find that the N- and C-terminal hydrophilic portions of the molecule independently contribute to correct targeting of this protein. The C-terminus functions to suppress expression of p64 on the plasma membrane and the N-terminus is necessary to direct p64 to its proper compartment.

MATERIALS AND METHODS

Tissue Culture

T84 and Panc1 cells were obtained from American Type Culture Collection (Rockville, MD). T84 cells were grown in 1:1 mixture of DMEM (low glucose) and Ham's F12 supplemented with 5% new-

born calf serum and 2% Ultraser G (IBF Biotechnics, Villeneuve-la-Garenne, France). Panc1 cells were grown in DMEM (low glucose) supplemented with 10% fetal bovine serum. DNA was transfected into Panc1 cells with Lipofectin (Life Technologies, Bethesda, MD) using the protocol provided by the manufacturer. Transformants were selected with 400 $\mu\text{g}/\text{ml}$ G418 or 200 $\mu\text{g}/\text{ml}$ hygromycin as appropriate.

Antibodies

Immunolocalization of p64 in T84 cells was performed using affinity-purified antisera which had been raised in a guinea pig against the native purified p64 (Redhead *et al.*, 1992). This antisera was affinity purified by selective binding to and elution from a lacZ-p64 fusion protein (Landry *et al.*, 1993) immobilized on nitrocellulose (Harlow and Lane, 1988). Immunolocalization of bovine p64 in transfected Panc1 cells was performed with antisera raised in rabbit using the lacZ-p64 fusion protein as antigen (Landry *et al.*, 1992). This antisera was affinity purified over lacZ-p64 fusion protein immobilized on an Aminolink column (Pierce, Rockford, IL). The OK-T4 hybridoma cell line was obtained from American Type Culture Collection for production of monoclonal antibody specific for CD4. Tissue culture supernatant was used to stain cells. Monoclonal antibodies to the transferrin receptor were obtained from Amersham (Arlington Heights, IL).

Immunofluorescence

Cells grown on glass coverslips to about 80% confluence were washed with ice-cold phosphate-buffered saline (PBS, 135 mM NaCl, 10 mM NaH_2PO_4 , pH 7.4) and immediately fixed with 100% methanol at -20°C for 5 min. Fixed cells were rinsed with PBS and then blocked for 1 h with 5% goat serum and 0.02% fish gelatin (Sigma, St. Louis, MO) in PBS (PBS-FG). Cells were incubated with primary antibody (undiluted affinity-purified guinea pig antibody, 1:10 dilution of affinity-purified rabbit antibody, or 1:2 dilution of hybridoma culture supernatant in PBS-FG) at room temperature for 1.5 h or at 4°C overnight. Cells were washed with four 15-min changes of PBS-FG and then incubated with appropriate secondary antibody diluted in PBS-FG. Cells were again washed with four exchanges of PBS-FG, the final wash of stained T84 cells going overnight at 4°C . When needed, cells were treated with 10 μM forskolin (Sigma), 1 μM A23187 (CalBiochem, Pasadena, CA), or 100 nM PMA (CalBiochem) in culture medium at 37°C in a 5% CO_2 incubator for the times indicated.

The fluid phase endocytic compartment was labeled with endocytosed fluorescent dextran. Cells were grown on coverslips as above and then incubated in culture medium supplemented with 10 mg/ml tetramethylrhodamine isothiocyanate (TRITC)-dextran (70 kDa) (Sigma) at 37°C for 30 min, washed for 90 min in medium at 4°C , and then fixed in paraformaldehyde-periodate-lysine (PLP, 2% paraformaldehyde, 75 mM lysine, 75 mM sodium phosphate, pH 7.0) for 45 min. Cells were then permeabilized for 5 min with 0.02% saponin in PBS, blocked with PBS-FG, and processed for staining with p64 antibodies as above.

Specimens were photographed on a Zeiss Axiophot fluorescent microscope using 63 $\times$ or 100 $\times$ oil immersion objectives as marked. Confocal microscopy was performed on an Olympus laser scanning microscope.

Immunoelectron Microscopy

Cells were fixed with PLP as above, permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS, and blocked with PBS-FG. Cells were incubated with affinity-purified antibody overnight, washed for 30 min at room temperature, and incubated with horse radish peroxidase (HRP)-goat anti-guinea pig (The Jackson Laboratory, Bar Harbor, ME) overnight at 4°C . HRP was developed with a 3,3'-diaminobenzidine substrate kit (Vector Laboratories, Burlin-

game, CA) and specimens were then postfixed with 1% osmium tetroxide for 1 h at room temperature. After dehydration by graded ethanol, cells were embedded in Epon resin. Ultrathin sections were counterstained with uranyl citrate and lead acetate and observed using a JEOL 2000FX electron microscope at 80 kV.

DNA Constructs

Maps of the various DNA constructs are shown in Figure 6. The cDNA insert from plasmid pNHΔP (Landry *et al.*, 1993) encoding the entire p64 was transferred to the eukaryotic expression vector, pcDNAneo (Invitrogen, Carlsbad, CA), putting expression of bovine p64 under control of the cytomegalovirus promoter and linked to a G418-selectable marker. Generation of C-terminal mutants, NHΔ71 and NHΔ73, was carried out by polymerase chain reaction (PCR) mutagenesis as follows. A sense oligonucleotide, H44 (5'-GTGAAGGCTGGAATCGATGGTGAA), which corresponds to positions 768–791 on the p64 sequence (Landry *et al.*, 1993), was used to amplify from pNHΔP DNA using one of two mutagenic oligonucleotides, H71 (5'-ACTGCAGCTAGGCCACGTCC) or H73 (5'-ACTGCAGTCAGGATTGGCTGAGGCT CTCGGCCACGTCC), corresponding to positions 1448–1439 and 1469–1439 on the p64 sequence, respectively, but encoding the desired mutations and with an added 5' *Pst*I site. Amplified products were cloned into PCRII vector (Invitrogen) and sequence determined using the Sequenase system (United States Biochemical, Cleveland, OH). The amplified fragment was then subcloned into the *Hinc*II-*Pst*I sites of NHΔP, and the constructs were again confirmed by sequence determination. Each construct was then transferred to pcDNAneo as above.

Construct NHΔH2B was generated by PCR mutagenesis from NHΔP as follows. A sense oligonucleotide, HM2 (5'-AGAGCTCATCTCAGACCTACTCG), corresponding to positions 104–119 of the p64 sequence with an added 5' *Sac*I site, was used to amplify from NHΔP with antisense mutagenic oligonucleotide HM1 (5'-ACCATTTCTCCTGGTCCTTGGTTGGTGAGAG), corresponding to positions 611–486 with positions 605 to 510 deleted. The PCR product was cloned as above. A plasmid with correct sequence was digested with *Sac*I and *Bst*XI, and the resulting fragment was inserted into *Sac*I- and *Bst*XI-digested pNHΔP, the identity of the resulting construct was again confirmed by DNA sequencing. The resulting construct encodes p64 with amino acids 119–150 deleted. The construct was then transferred to the pcDNAneo expression construct as above.

The plasmid pBSCD4, containing the *Eco*RI to *Bam*HI fragment of plasmid pT4B (Maddon *et al.*, 1985) inserted into the *Eco*RI-*Bam*HI sites of pBluescript SK⁻ (Stratagene, La Jolla CA), was a gift from Andrey Shaw (Department of Pathology, Washington University, St. Louis, MO). To generate CD4-p64 chimeras, pBSCD4 was digested at the unique *Nar*I site, the ends filled in with T4 DNA polymerase, digested at the flanking *Xba*I site, and the large fragment containing the vector sequences and the N-terminal coding region of CD4 were isolated. pNHΔP was digested at the unique *Xma*I site, the ends filled in as above, and then digested at the flanking *Xba*I site, and the fragment containing the region encoding the C-terminus of p64 was isolated. The fragments were ligated and used to transfect bacteria. The resulting plasmid encodes the entire CD4 protein up to amino acid 404 (numbered from the N-terminus of the mature protein) followed by the final 28 amino acids of p64. The CD4Δ71 chimera was generated in an identical manner starting with pNHΔ71 instead of pNHΔP.

To generate construct pBSCD4H2B, pBSCD4 was cut with *Nar*I, made blunt with T4 DNA polymerase, cut with *Bam*HI, and the large vector/CD4 containing fragment was isolated. cDNA clone H2B (Landry *et al.*, 1993) contains the entire p64 coding region, except the initiating methionine with a *Sma*I restriction site in the plasmid vector sequence immediately upstream of the p64 coding sequences. H2B was digested with *Sma*I and *Bgl*III and the 550-bp fragment encoding the N-terminus of p64 was isolated. The isolated

fragments were ligated and recombinant plasmids cloned by transformation of bacteria. The resulting construct encodes CD4 up to amino acid 404 followed by nine amino acids encoded by the polylinker and then amino acids 2 through 178 of p64 followed immediately by a stop codon. The identity of the resulting construct was confirmed by DNA sequence. The coding regions of pBSCD4, pBSCD4ΔP, pBSCD4-Δ71, and pBSCD4H2B were each transferred to the multiple cloning site of pCEP4 (Invitrogen), putting each construct under control of the cytomegalovirus promoter and linked to a hygromycin-selectable marker.

Oocyte Expression Studies

Oocyte expression constructs were generated by transferring the coding regions of plasmids pNHΔP, pNHΔ71, and pNHΔ73 into the multiple cloning site of the oocyte expression vector, pSD64TR (Krieg and Melton, 1984). The plasmid was linearized and capped RNA was prepared using SP6 RNA polymerase. RNA was injected into defolliculated oocytes and oocytes were incubated for 3 days at 15°C. Intact, healthy-appearing oocytes were selected for biotinylation studies. Oocytes were gently rinsed in amphibian ringers (90 mM NaCl, 7 mM NaHPO₄, 1 mM CaCl₂, 1 mM MgCl₂) and then incubated in amphibian ringers with 0.5 mg/ml NHS-LC-biotin (Pierce, Rockford, IL) for 30 min at 4°C. The reaction was quenched by gently rinsing the oocytes with ice-cold 50 mM NaCl/50 mM Tris (pH 8.0) for three exchanges, 3 min each. Oocytes were then suspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 8, 50 μM phenylmethylsulfonyl fluoride) and homogenized in a Teflon Dounce homogenizer on ice. Insoluble material was removed by centrifugation at 15,000 rpm for 5 min at 4°C in a microfuge, and the protein concentration of the supernatant was determined with BCA reagent (Pierce). From each group of oocytes, 200 μg of protein were then incubated with 25 μl of a 50% suspension of avidin-agarose beads (Pierce) for 30 min at room temperature. The beads were collected by centrifugation at 7000 rpm for 2 min, then washed four times with lysis buffer, and suspended in 30 μl of loading buffer. The entire sample associated with avidin beads was run next to 50 μg of total protein from each group of oocytes. Samples were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose. The blot was stained with Ponceau S to determine the position of molecular weight standards, probed with affinity-purified rabbit anti-p64, alkaline-phosphatase-conjugated secondary antibody, and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium using standard methods (Harlow and Lane, 1988).

RESULTS

p64 Is Located in Vesicles Resembling Secretory Granules

Affinity-purified antibodies recognizing p64 were used to probe rabbit kidney tissue reactions as shown in Figure 1A. Proximal tubular cells stained most intensely, showing an intracellular punctuate pattern consistent with intracellular vesicles. The p64 staining is most prominent in the perinuclear region of the cell.

We also studied p64 expression in the human colon cancer cell line T84 (Dharmasathaphorn *et al.*, 1984). We had previously shown that T84 cells express p64 at protein (Redhead *et al.*, 1992) and mRNA (Landry *et al.*, 1993) levels. However, immunolocalization in T84 cells has been hampered by the relatively low abundance of the protein in these cells. To overcome this problem, we searched for culture conditions that would optimize p64 expression. We found that cultur-

Figure 1. Immunolocalization of p64. (A) Frozen section of rabbit kidney probed with affinity-purified guinea pig anti-p64 antibodies/FITC-conjugated goat anti-guinea pig. An area of proximal tubule is shown; the arrow marks the apical membrane of a proximal tubule cell. (B) T84 cells grown on a glass coverslip and probed as above. Cells photographed under 63 $\times$ objective. Bar, 10 μ m.

ing the cells in fetal calf serum plus 2% of the serum substitute Ultraser G increased the abundance of this protein by more than 10-fold. This effect was partially mimicked by growth in the presence of new born calf serum.

Affinity-purified anti-p64 antibodies were used to localize the protein by indirect immunofluorescence microscopy in Ultraser G-treated T84 cells. Anti-p64 labeled central intracellular vesicles (Figure 1B). To determine the identity of the p64-containing vesicles, p64 immunofluorescence was compared with that of two endocytic markers: transferrin receptor which defines a "recycling compartment," and endocytosed fluorescent dextran which labels the fluid phase endocytic pathway (Figure 2). Double-labeled cells were viewed by taking optical sections using confocal light microscopy. Three sections from cells labeled with both anti-p64 (fluorescein:green) and antitransferrin receptor (rhodamine:red) are shown in Figure 2 (A–C). The levels of these sections are depicted in Figure 2D. The section in Figure 2A was taken from the basal end of the cells and shows a wide distribution of the transferrin receptor with a few green-labeled p64 vesicles. The next section is one taken about 4 μ m from the basal surface and shows p64-containing vesicles grouped in large clusters. The third section (Figure 2C) taken 2–4 μ m from the apical surface shows large, evenly distributed transferrin receptor-associated vesicles interspersed with a few p64 vesicles. There is also evidence of some yellow fluorescence, indicating that there may be a limited amount of colocalization in this apical region. Sections in Figure 2, E and F, are equivalent sections to those in B and C through cells colabeled with endocytosed rhodamine-labeled dextran (red) and anti-p64 (green), showing a few dextran-labeled vesicles peripheral to the basal groups of p64 vesicles and a greater number of dextran-labeled vesicles toward the apical surface interspersed with a few p64 vesicles. Thus, p64 is expressed in a vesicular pattern concentrated at the basal pole of T84 cells which is distinct from either the fluid phase or receptor-mediated endocytic compartments.

Electron microscopic studies revealed that situated in the basal part of T84 cells were groups of vesicles, 0.5–1.0 μ m in diameter, filled with a uniform somewhat electron-dense material (Figure 3A). These vesicles contained p64 in their membranes, as demonstrated by immunoperoxidase labeling (Figure 3B). We did not detect staining for p64 in any other cell structure. Like p64, these vesicles were only present in abundance after treatment of the cells with Ultraser G. The contents of these vesicles appeared to be carbohydrate rich, since they were only observed following fixation with the carbohydrate cross-linking fixative PLP (McClellan and Nakane, 1974). At the light microscopic level, vesicles with identical distribution show amylase-resistant periodic acid-Schiff (PAS) staining. The combination of selective fixation with a carbohydrate-cross-linking fixative and amylase-resistant PAS

Figure 2. Confocal colocalization of p64 and endocytic compartments in T84 cells. (A–D) T84 cells were fixed and stained with affinity-purified guinea pig anti-p64/FITC-conjugated goat anti-guinea pig (green) and mouse monoclonal anti-transferrin receptor/TRITC-conjugated goat anti-mouse (red). The stained cells were visualized under confocal microscopy and images were collected at three different levels as diagrammed in D. A is at the extreme basal pole of the cell, B is about 4 μm from the basal pole, and C is subapical. (E and F) T84 cells were allowed to endocytose TRITC-dextran (red) and then fixed and stained with affinity-purified anti-p64/FITC-conjugated goat anti-guinea pig. Cells were visualized as above. Planes of focus for E and F correspond to levels of B and C, respectively.

Figure 3. Characterization of p64 compartment in T84 cells using electron microscopy. (A) Electron micrograph of unstained T84 cells demonstrating the large basal vesicles filled with homogeneous, moderately electron-dense material. (B) T84 cells were fixed with PLP, permeabilized with Triton X-100, and stained with affinity-purified guinea pig anti-p64/HRP-conjugated goat anti-guinea pig, developed with DAB, and processed for electron microscopy as described in MATERIALS AND METHODS. Because of the Triton X-100 permeabilization and lack of glutaraldehyde fixation, tissue preservation is not as good as in A. However, the limiting membranes of the large basal vesicles filled with homogeneous, moderately electron-dense material clearly stain for p64 (arrowhead). The apical surface of both panels is at the top. Bar, 1 μ m.

staining indicates that these vesicles may contain large amounts of proteoglycans or mucins.

p64 Is Located in a Second Messenger-regulated Secretory Pathway

In T84 cells, regulated exocytosis stimulated by both protein kinase C (PKC) and intracellular calcium takes place both at the apical and basolateral membranes (Huflejt *et al.*, 1994). We asked whether p64 vesicles belong to a secretory pathway by monitoring the distribution of p64 following treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA). Within 5 min of stimulation with 100 nM PMA, p64 immunofluorescence had moved from the cell center (Figure 4A, untreated cells) toward the cell periphery (Figure 4B, PMA-treated cells). The immunofluorescence was often located in what appeared to be fused or aggregating groups of vesicles toward the cell periphery. An identical response was elicited by treatment of the cells with the calcium ionophore A23187 (10 μ M) for 30 min. The changes in distribution were

not due to changes in cell morphology or confluency since the pattern of transferrin receptor distribution was unaffected. Reagents such as forskolin that elevate cyclic AMP also stimulate exocytosis in T84 cells, but only apically (Huflejt *et al.*, 1994). Treatment of the cells with forskolin did not cause a marked change in the distribution of p64 by immunofluorescence. These data are consistent with the presence of p64 in a secretory pathway which is regulated by both calcium and PKC but not by cyclic AMP.

Determinants of Subcellular Distribution of p64

The immunolocalization results above imply rigorous targeting of p64 to a distinct class of secretory vesicles. Little is known regarding targeting of integral membrane proteins to secretory vesicles. We sought to identify structural features of p64 which determine subcellular distribution using mutational analysis in a heterologous expression system.

As a model system for expression of p64, we used the human pancreatic cancer cell line Panc1 (Lieber *et*

Figure 4. Redistribution of p64 in T84 cells following stimulation by PMA. T84 cells were grown on coverslips and exposed to control medium (A) or medium with 100 nM PMA (B) for 15 min at 37°C and then fixed and stained as in Figure 1. Cells were photographed under 63× objective. Bar, 10 μ m.

al., 1975) which does not express detectable endogenous p64 at protein or mRNA levels. We transfected Panc1 cells with the expression construct, NH Δ P (Figure 6B), encoding bovine p64 and selected stable transformants. Cells were stained with affinity-purified anti-p64 antibodies as shown in Figure 5. Transformants expressed p64 in a prominent peripheral reticular-vesicular pattern (Figure 5A). The distribution of p64 did not change when cells were exposed to 10 μ M forskolin prior to fixation (Figure 5B). However, p64 dramatically changed distribution in response to calcium ionophore (Figure 5C) or phorbol ester (Figure 5D), assuming a pattern more consistent with cell surface expression. There was no apparent change in cell shape in response to forskolin or phorbol ester but unlike our results with T84 cells, there was a prominent shrinkage of the Panc1 cells in response to calcium ionophore (Figure 5C). These results are representative of four independent stably expressing cell lines. No signal is detectable when the parent Panc1 cells are stained with the p64 antibodies (see Figure 7E). Thus, exogenous p64 is expressed in a reticular-vesicular compartment of Panc1 cells which is regulated similarly to the vesicular compartment of endogenous p64 in T84 cells.

As an initial approach to definition of targeting signals in p64, we examined the domains of p64 which are thought to be cytoplasmic. The only data regarding membrane topology of p64 are from the hydrophathy analysis of the amino acid sequence (Landry *et al.*, 1993). Based on this data, p64 is predicted to consist of a 200-amino acid cytoplasmic domain at the N-terminus followed by a transmembrane segment, a 127-amino acid extracytoplasmic domain, a second transmembrane segment, and a C-terminal 53-amino acid cytoplasmic domain. Within the N-terminal hydrophilic domain lies several consensus phosphorylation sites for PKC and a multiply repeated, proline-rich, acidic motif of unknown function. Within the C-terminal hydrophilic domain lies the single consensus protein kinase A phosphorylation site of the molecule. We independently studied the N- and C-terminal domains of p64 to determine whether these sequences contribute to subcellular distribution of p64.

Role of the C-Terminus in Targeting. We were particularly interested in the C-terminus of p64 since we noted similarity with the endoplasmic reticulum (ER) retrieval sequence (Jackson *et al.*, 1990; Nilsson and Warren, 1994) in which positively charged amino acids at positions 3 and 4 (or 5) from the C-terminus

Figure 5. Expression of bovine p64 in Panc1 cells. Cells from a stable transfected Panc1 cell line expressing bovine p64 were fixed and stained with affinity-purified rabbit anti-p64/FITC-conjugated goat anti-rabbit following exposure to the following supplemented media at 37°C for 15 min: A, control medium; B, 10 μ M forskolin; C, 100 nM PMA; and D, 1 μ M A23187. Cells were photographed under 100 $\times$ objective.

confers ER localization of membrane proteins. The p64 sequence, with positively charged amino acids at positions -2, -5, and -6 (Figure 6A), only loosely resembles the ER motif and would not be expected to function as an efficient ER retrieval signal. To determine whether the C-terminus is important in subcellular targeting of p64, we generated two mutants in which this sequence has been altered, as shown in Figure 6A. In mutant NH Δ 71, the last six amino acids were deleted while in mutant NH Δ 73, the positively charged amino acid at positions -2, -5, and -6 have been mutated to neutral or negatively charged residues.

Panc1 cells were transfected with expression constructs encoding p64 or either of the two C-terminal mutants. Transfected cells were stained with affinity-purified antibodies directed against p64. Cells expressing wild-type p64 stain in the peripheral vesicular pattern described above (Figure 7A). Cells transfected with mutant NH Δ 71 (Figure 7B) or NH Δ 73 (Figure 7C) stain in a pattern consistent with cell surface expression. As the NH Δ 71 and NH Δ 73 transfor-

ants were continued in culture through three or four passages, surface expression of the mutant constructs was lost. In fact, most cells staining strongly for expression were rounded up and appeared to be dying (Figure 7D), suggesting a selection against surface expression of this protein. Untransfected Panc1 cells do not stain with affinity-purified anti-p64 antibodies (Figure 7E).

Expression was also assessed in *Xenopus* oocytes. Capped RNA encoding wild-type p64 or the NH Δ 71 or NH Δ 73 mutants was injected into oocytes. On the third day, plasma membrane proteins were labeled with NHS-LC-biotin, a membrane impermeant-biotinylating reagent. The oocytes were then solubilized and allowed to bind to avidin-agarose. Samples of the total oocyte lysates and those proteins which were bound to avidin-agarose were separated by SDS-PAGE and probed with affinity-purified anti-p64 antibodies as shown in Figure 8. Proteins expressed on the cell surface should be accessible to the biotinylating agent and thus be present in the avidin-precipitated material, whereas those proteins excluded from

the plasma membrane should not. The results clearly demonstrate that wild-type p64 is excluded from the plasma membrane of oocytes whereas the mutants NH Δ 71 and NH Δ 73 are expressed on the plasma membrane. Thus, mutation or deletion of the C-terminus of p64 leads to aberrant membrane targeting and plasma membrane expression of p64.

To determine whether the C-terminus of p64 is sufficient to function as a signal for targeting to its vesicular compartment, we created an expression construct encoding a chimeric protein in which the C-terminus of a natural plasma membrane protein is replaced with the C-terminus of p64 as diagrammed in Figure 6B. We used human CD4 as a model plasma membrane protein. CD4 is a normal cell surface protein of certain lymphocytes and is not expressed by Panc I cells. This protein is known to have a simple topology with an extracellular N-terminus, a single transmembrane segment, and a cytoplasmic C-terminal domain of 40 amino acids (Maddon *et al.*, 1985). Using naturally occurring restriction sites, we replaced the C-terminal 32 amino acids of CD4 with the C-terminal 28 amino acids of wild-type p64 or the corresponding region of the NHΔ71 mutant.

Wild-type CD4 or the CD4-p64 chimeras were transfected in Panc I cells and stable cell lines selected. Cells were stained with monoclonal antibody against CD4 as shown in Figure 9. The wild-type CD4 is expressed in a plasma membrane distribution (Figure 9A), and the CD4-p64 chimera shows a staining pattern consistent with a prominent intracellular membrane distribution that is distinct from the vesicular staining pattern of p64 itself (Figure 9, B and C). Labeling of cells with membrane impermeant-biotinylating reagents followed by avidin affinity purification demonstrates that the chimeric protein is not quantitatively excluded from the plasma membrane (unpublished observation). Expression of the CD4- Δ 71 chimera is similar to that of wild-type CD4 (Figure 9D). Thus, as observed with p64, the C-terminus functions to depress cell surface expression of the CD4-p64 chimera, and this effect is removed by deletion of the terminal six amino acids.

The distribution of the CD4-p64 chimera is clearly distinct from that of both wild-type CD4 and wild-type p64. Thus, while the C-terminal sequence of p64 apparently suppresses the expression of CD4 on the plasma membrane, it does not confer the characteristic distribution of p64, suggesting additional targeting signals may exist.

Role of the N-Terminus in Targeting. We asked whether the N-terminal domain of p64 plays a role in targeting. Within the N-terminal domain at amino acids 117–149 is a proline-rich, acidic motif (QASD-PEEP) that is directly repeated four times. The function of this domain is not known, although it contains the essential PXXP motif which binds SH3 domains of

A

⁴²⁸
 p64 . . . A D V A K R L S R S_{COO}
 Δ71 . . . A D V A_{COO}
 Δ73 . . . A D V A E S L S Q S_{COO}

B

Figure 6. p64 expression constructs. (A) C-terminal amino acid sequences of p64 and the $\Delta 71$ and $\Delta 73$ mutants. Each sequence begins at amino acid 428 of p64 and extends to the C-terminus. (B) Maps of cDNA constructs. Thick open or hatched bars indicate the coding regions of p64 (hatched) or CD4 (open) cDNAs. Thin lines indicate flanking, noncoding cDNA sequences. The thick filled lines denote flanking plasmid vector sequences. Regions encoding hydrophobic putative transmembrane segments are boxed. The region encoding the proline-rich, acidic repeated motif is denoted by the overlined segment. The positions of oligonucleotide primers used in mutagenesis are denoted by the arrows beneath the pNHAP map. Relevant restriction sites shown are B, *Bst*XI; Ba, *Bam*HI; Bg, *Bgl*II; H, *Hinc*II; N, *Nar*I; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; X, *Xma*II; Xb, *Xba*I. The (Sm) indication on the NHAP map marks the position of the *Sma*I site in the related plasmid, H2B. The sites in parenthesis on the lower maps indicate where restriction sites were eliminated by filling in and/or ligation. For clarity, the maps are not precisely to scale.

proteins related to the Src tyrosine kinase (Alexandropoulos *et al.*, 1995; Mayer and Eck, 1995). We generated a mutant, NHΔH2B, in which this repeated domain is deleted from p64 (see Figure 6B). Expression of this construct in Panc1 cells (Figure 10A) leads to a dramatically different subcellular distribution than naturally occurring p64 (compare with Figures 5A and 7A). Rather than the characteristic peripheral reticular-vesicular pattern of p64 in Panc1 cells, NHΔH2B is expressed in a diffuse perinuclear pattern.

To determine whether the N-terminal domain is sufficient for targeting, we created a chimeric expression construct in which the final 30 amino acids of CD4 were replaced with amino acids 2 through 178 of p64. This construct, CD4-H2B (see Figure 6B), was transfected into Panc1 cells and stable cell lines selected. Cells were stained with monoclonal antibody

Figure 7. Expression of C-terminal mutant p64 constructs in Panc1 cells. Stable cell lines expressing normal p64 and each of the $\Delta 71$ and $\Delta 73$ mutants were fixed and stained as described in Figure 5 legend. (A) Cells expressing NH Δ P, encoding normal bovine p64. (B) Cells expressing NH $\Delta 71$. (C) Cells expressing NH $\Delta 73$. (D) Cells expressing NH $\Delta 71$. For both NH $\Delta 71$ - and NH $\Delta 73$ -expressing cell lines, those cells with the most intense staining characteristically appeared rounded-up and dying like these. (E) Untransfected Panc1 cells, stained, photographed, and processed identically to A–D. Cells were photographed under 100 $\times$ objective.

against CD4 (Figure 10C). The CD4-H2B chimera is expressed in a vesicular pattern similar to the pattern of expression of p64 itself. Unlike expression of p64, however, a sizable fraction of CD4-H2B is also present in the cell surface pattern and is accessible to biotinylation agents (unpublished observation). Stimulation

of cells with PMA does not lead to marked redistribution of the chimera, although this is difficult to assess since a fraction of the protein is already distributed in a cell surface pattern. Thus, the N-terminus of p64 confers targeting to a subcellular compartment similar to that of normal p64. However, the targeting is inefficient, with a significant fraction of the protein escaping to the cell surface.

Figure 8. Surface expression of p64 constructs in *Xenopus* oocytes. Western blot of 50 μ g of total protein (T) or surface biotinylated material from 200 μ g of total solubilized oocyte protein (B) from control oocytes (C) or oocytes expressing p64, $\Delta 71$, or $\Delta 73$ mutants probed with affinity-purified anti-p64 antibodies. The 64-kDa band in the T lanes demonstrates successful expression of each construct; a band in the B lanes indicates exposure of that construct to extracellular media (i.e., plasma membrane expression).

DISCUSSION

In this article, we have described the intracellular distribution of the microsomal chloride channel protein p64 and investigated structural features which determine targeting of this protein to its vesicular compartment.

Subcellular Distribution of p64

In rabbit kidney proximal tubule and cultured human intestinal epithelial cells, p64 is expressed in a perinuclear vesicular pattern. Confocal microscopy studies of cells colabeled with p64 antisera and markers of the fluid phase and recycling endosomal pathways demonstrate that p64-containing structures are distinct from the endosomal compartments and are concentrated in the basal pole of T84 cells. Electron microscopy demonstrates that p64 is localized to distinct dense-core vesicles. Finally, p64-containing vesicles redistribute in response to phorbol ester or calcium

Figure 9. Pancl cells expressing CD4/P64 chimeras. Stable Pancl cell lines expressing CD4, CD4 Δ P, or CD4 Δ 71 were selected, fixed, and stained with OKT4 monoclonal antibody/FITC-conjugated goat anti-mouse. (A) Cells expressing normal CD4. (B and C) Cells expressing CD4 Δ P. (D) Cells expressing CD4 Δ 71. Cells were photographed under 100 $\times$ objective.

ionophore but not in response to forskolin. We conclude that p64 is selectively expressed in a distinct population of regulated secretory vesicles.

The identity of the material in these vesicles is unknown. Our observations that these vesicles are only observed when cells are fixed with PLP and that the vesicles demonstrate amylase-resistant PAS staining suggests that the contents may be rich in glycoproteins, glycosaminoglycans, or mucins. T84 cells are known to demonstrate regulated secretion of mucins in culture (McCool *et al.*, 1990). Furthermore, mucin secretion is stimulated by phorbol esters and by calcium ionophores. However, mucin is thought to be primarily secreted apically whereas the p64-containing vesicles appear to move to a basolateral distribution upon stimulation. Another candidate for the content of p64 vesicles would be extracellular matrix proteins which are heavily glycosylated.

Two distinct roles for anion channels in exocytosis have been firmly established in a few model systems. First, anion channels are necessary for acidification of some secretory vesicles in which the low luminal pH has been implicated in vesicle loading or in processing of vesicle contents. Second, regulated anion channels are present in nonacidifying secretory vesicles of pancreas and salivary glands where they are thought to function in conjunction with potassium channels to cause osmotic swelling of the vesicles prior to exocytosis. Perhaps p64 carries out one of these roles for this class of secretory vesicles.

Determinants of Subcellular Distribution of p64

We had previously noted that expression of p64 in *Xenopus* oocytes is excluded from the plasma membrane (Landry *et al.*, 1993). Here, we have shown that p64 is selectively targeted to a specific class of intracellular vesicles. A variety of mechanisms have been proposed to account for targeting of membrane proteins to correct subcellular domains. In many cases, structural motifs within the cytoplasmic domains of membrane proteins have been implicated as targeting signals. We examined the predicted cytoplasmic domains of p64 to identify structures which could be responsible for targeting of p64 to its intracellular compartment.

We first analyzed the C-terminal hydrophilic domain of p64 for targeting signals. We generated two mutants of p64 in which the C-terminal sequence is disrupted. Unlike normal p64, these mutant constructs are not expressed in the characteristic reticular-vesicular pattern in Pancl cells, but are expressed in a distribution consistent with the plasma membrane. When expressed in *Xenopus* oocytes, the C-terminal mutants are accessible to impermeable biotinylating reagents whereas normal p64 is not, proving that the mutant proteins are in fact exposed to the external face of the plasma membrane.

The importance of the extreme C-terminus in membrane targeting was further supported by our studies of CD4-p64 chimeras. Substitution of the normal CD4

Figure 10. N-terminal domain is necessary for correct targeting of p64 and confers p64 targeting to CD4. (A) Panc1 cells expressing NHΔH2B construct were fixed and stained as described in Figure 5 legend. (B) Identical cell line in A stained with control antibody (affinity-purified rabbit anti- β -galactosidase), developed with FITC-conjugated goat anti-rabbit, photographed, and processed identically to A. (C) Panc1 cells expressing CD4H2B, fixed, and stained as described in the legend to Figure 9. Cells were photographed under 100 $\times$ objective. Bar, 10 μ m.

C-terminus with that of p64 results in a protein whose distribution is shifted so that a significant fraction of the staining is in an intracellular pattern. Nonspecific disruption of protein structure may lead to retention of membrane proteins in the ER (Pelham, 1989; Cheng *et al.*, 1990). The observation that retention of CD4-p64 chimeras in internal membranes is dependent on the last six amino acids of p64 indicates that our observations are unlikely to be due to nonspecific effects on folding of CD4. Thus, in this model system, the extreme C-terminus of p64 functions to suppress expression of the protein on the cell surface and increase expression in an intracellular membrane fraction. The C-terminus of p64 clearly does not confer the normal p64 distribution onto CD4.

We examined the N-terminal hydrophilic domain of p64 for the presence of targeting information and found that deletion of the multiply repeated motif from the N-terminal domain of p64 disrupts sorting of p64, leading to expression in a diffuse perinuclear pattern. In a complementary experiment, the N-terminal domain of p64 was used to replace the C-terminal domain of CD4. We found that the p64 N-terminus confers sorting of CD4 to an intracellular vesicular domain similar to that of native p64. This sorting is inefficient in that a sizable fraction of the CD4-H2B chimera escapes to the plasma membrane. The impli-

cation of the repeated proline-rich sequence in membrane targeting is particularly interesting since this proline-rich motif contains the PXXP consensus for SH3-binding domains (Alexandropoulos *et al.*, 1995; Mayer and Eck, 1995). SH3 interactions have been shown to function in sorting of at least one ion channel (Rotin *et al.*, 1994). Whether the repeated proline-rich motif of p64 is an SH3-binding domain remains to be determined. Furthermore, our experiments do not address whether sequences in the N-terminal domain of p64 other than the proline-rich motif are involved in targeting.

These data indicate that at least two physically distinct portions of p64 are necessary for high-fidelity targeting of p64. The N-terminal domain of p64 functions as a positive targeting signal: disruption of a portion of this sequence eliminates targeting to the proper compartment while introduction of this sequence into an unrelated plasma membrane protein confers characteristic p64 targeting to the chimeric protein, albeit inefficiently. In contrast, the C-terminal domain seems to act as a negative targeting signal in that it prevents expression of p64 in the plasma membrane, but does not actively target the protein to its proper destination. Thus, it appears that the C-terminal domain of p64 functions as a retention or a retrieval sequence, keeping p64 off the plasma mem-

brane and allowing the N-terminal targeting signal to direct p64 to its final destination efficiently.

Our data do not differentiate between the C-terminus acting as a retention signal keeping p64 in some internal membrane compartment or a retrieval signal, causing p64 to be rapidly internalized if expressed on the surface. Plasma membrane internalization signals have been described which are critically dependent on a tyrosine residue in the cytoplasmic domain of membrane proteins. There is a tyrosine residue at position -11 from the C-terminus of p64 whose immediate environment fits the general consensus for a type I internalization signal (Naim and Roth, 1994). Our C-terminal mutations of p64 do not impinge on the consensus internalization signal. Whether the targeting effects of our C-terminal mutations are the result of disruption of a higher order structure of a tyrosine internalization signal remains to be investigated.

Recently, targeting signals for peptidylglycine α -amidating monooxygenase (PAM), an integral membrane protein which is expressed partially in regulated secretory vesicles of neuroendocrine cells, have been studied (Milgram *et al.*, 1996). One significant difference between PAM and p64 is that the steady-state distribution of PAM is such that a substantial majority of membrane-associated PAM is present in the trans-Golgi network and not in secretory vesicles. Nonetheless, similar to our results, these authors found that targeting information for PAM could be dissected into two components, one necessary to prevent surface expression and one necessary for delivery to secretory vesicles. We find no significant homology between the amino acid sequences of PAM and p64, particularly in the regions implicated in targeting.

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