Retention of p63 in an ER–Golgi Intermediate Compartment Depends on the Presence of All Three of Its Domains and on Its Ability to Form Oligomers

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Abstract. The type II membrane protein p63 is a resident protein of a membrane network interposed between rough ER and Golgi apparatus. To study the retention of p63, mutant forms were expressed in COS cells and the intracellular distribution determined by immunofluorescence microscopy. Investigation of chimeric constructs between p63 and the plasma membrane protein dipeptidylpeptidase IV showed that protein sequences from all three domains of the p63 protein are required to achieve complete intracellular retention. Mutational analysis of the 106-amino acid cytoplasmic tail of p63 revealed that the NH₂-terminal 23 amino acids are necessary for retention. When p63 was solubilized with Triton X-100 and subjected to centrifugation at 100,000 g, it formed large, insoluble oligomers, particularly at neutral pH and below. A comparison of the behavior of wildtype and mutant p63 proteins in this assay revealed a perfect correlation between the formation of large oligomers and correct intracellular retention. These results suggest that self-association may be a major mechanism by which p63 is retained between the rough ER and the Golgi apparatus.

The secretory pathway of eukaryotic cells is composed of a series of different compartments through which newly synthesized proteins are transported en route to their final destination in the cell (Palade, 1975). Each of the subcellular organelles along this pathway contains a characteristic set of proteins that maintain their structural and functional integrity. A challenging question in contemporary cell biology is to understand how resident components, unlike itinerant proteins, achieve specific localization in a given intracellular compartment.

It is currently believed that secretory proteins are transported by a default pathway while resident proteins are selectively retained in individual organelles by means of specific signals (Pfeffer and Rothman, 1987; Wieland et al., 1987; Karrenbauer et al., 1990). Two types of signals, which mediate either the retention or the retrieval of proteins, have been identified to date. A variety of luminal ER proteins are retained in the cell by means of a COOH-terminal tetrapeptide (KDEL) or related sequence (Munro and Pelham, 1987; reviewed by Pelham, 1989) that allows retrieval of the proteins from a post-ER site by means of KDEL receptors (Lewis and Pelham, 1990). A group of type I transmembrane proteins of the ER are also retained in this organelle by a retrieval mechanism (Jackson et al., 1993). These proteins contain cytoplasmic sequences consisting of two lysine residues positioned three and four or five residues from the COOH terminus (KXXX or KXKXX, where X can be almost any amino acid) (Jackson et al., 1990; Shin et al., 1991). Recycling of these proteins is believed to occur from multiple post-ER locations along the secretory pathway (Jackson et al., 1993). In addition, retrieval has been suggested to be the mechanism by which the integral membrane protein TGN38 is targeted to the TGN (Bos et al., 1993; Humphrey et al., 1993). TGN38 possesses a tyrosine-containing motif within its cytoplasmic tail that is both necessary and sufficient for TGN localization (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). In contrast to these examples, localization of Golgi proteins is achieved by retention rather than retrieval. The retention of several Golgi proteins has been shown to depend primarily on their single transmembrane segment with some additional contribution to retention by the sequences just adjacent to those membrane anchors (Nilsson et al., 1991; Swift and Machamer, 1991; Machamer et al., 1993). In the case of the α 2,6-sialyltransferase, the cytoplasmic and luminal sequences flanking the transmembrane segment appear to be the crucial elements for retention rather than the transmembrane segment itself (Munro, 1991; Dahdal and Colley, 1993). It has been postulated that resident Golgi proteins oligomerize in the Golgi apparatus upon recognition of identical or related...
proteins, forming a complex of sufficient size to prevent entry into transport vesicles (Machamer, 1991; Nilsson et al., 1991, 1993, 1994). Recently, oligomerization of a chimeric protein containing the first membrane-spanning domain of the M glycoprotein of avian coronavirus has been correlated to its retention in the Golgi apparatus (Weisz et al., 1993).

In contrast to the considerable progress that has been made in understanding protein localization in the ER and the Golgi apparatus, it is presently unclear how the recently identified ER-Golgi intermediate compartment (ERGIC) maintains its resident population of proteins (for a review see Hauri and Schweizer, 1992). The ERGIC comprises a membrane system interposed between the rough ER and the cis side of the Golgi apparatus that has been shown to mediate ER-to-Golgi exocytic protein transport (Schweizer et al., 1990; Lotti et al., 1992). The ERGIC may correspond to the "budding compartment" of mouse coronavirus (Tooze et al., 1988; Krijnse-Locker et al., 1994) and to the "pre-Golgi vacuoles" of Semliki Forest virus-infected cells (Saraste and Kuismanen, 1984). Isolation of the ERGIC from Vero cells indicated that this compartment has unique properties since it does not share major marker proteins with its direct neighbor organelles, the rough ER and cis-Golgi apparatus (Schweizer et al., 1991). Currently, a limited number of marker proteins are available for the ERGIC. The ERGIC was originally defined by a 53-kD type I transmembrane protein (Schweizer et al., 1988) termed ERGIC-53. ERGIC-53 is specifically localized in the ERGIC at 37°C but undergoes a temperature-dependent redistribution (Lippincott-Schwartz et al., 1990; Schweizer et al., 1990) which suggested that it may follow a recycling pathway (Hauri and Schweizer, 1992). Another marker for the ERGIC is the small GTP-binding protein rab2p (Chavrier et al., 1990). Beta-COP, a major component of non-clathrin-coated vesicles, also associates with the ERGIC at 15°C (Dudenhoven et al., 1991). The p58-positive pre-Golgi elements described by Saraste and Svenson (1991) may also be part of the ERGIC.

The only membrane protein characterized and cloned so far with a stable ER-Golgi intermediate localization is p63 (Schweizer et al., 1993a,b). mAbs against p63 recognized an extended ER-Golgi intermediate membrane structure which indicated that the ERGIC may be larger than previously suggested by the ERGIC-53 analysis. Unlike ERGIG-53, the distribution of p63 was insensitive to organelle perturbants such as low temperature and brefeldin A (Schweizer et al., 1993a). Sequence analysis together with biochemical data demonstrated that p63 is a nonglycosylated, reversibly palmitoylated type II transmembrane protein with a 106 amino acids (Schweizer et al., 1993a,b). The p63wt cDNA was as described previously (Schweizer et al., 1993b) and consisted of the 5' untranslated region, bp5-84, the 1803 nucleotide coding region and 1023 bp of the 3' non-coding sequence. The full-length cDNA was inserted into the EcoRI site of the pECRI site of the Bluescript SK- vector (Stratagene, La Jolla, CA), respectively, with the initiation ATG facing the BamHI restriction site of the polylinker. The resulting constructs were designated pBSK-p63 or pbKS-p63, respectively. For transient expression in COS cells, the p63 insert was subcloned into the EcoRI site of the pCEEC vector (kindly provided by Dr. M. Spiess, Biozentrum, Basel, Switzerland) (Ellis et al., 1986) to give plasmid pCEEC-p63.

The mutant forms of the p63 gene were created using standard PCR protocols (Ho et al., 1989). All mutants start at bp78 of the original wild type (wt) p63 cDNA (Schweizer et al., 1993b). The final PCR products were digested with BamHI and used to replace the BamHI-BamHI fragment of pBSK-p63. The entire PCR-derived fragment was sequenced by the dideoxy termination procedure (Sanger et al., 1977) as described (Schweizer et al., 1993b). Correct clones were subcloned into the EcoRI site of the pCEEC vector for transient expression in COS cells.

To generate the A2-101 construct (p63 with deletion of amino acids 2-101), pBSK-p63 was assembled as a template to the 5' primer GCC GCG CAT GCG CAG CCT GCG and the 3' primer GCC CAA CAC CAC, using the 5' primer CAC CCT GCG CAG and an additional 3' primer GCC CAA CAC CAC. A subsequent PCR reaction, the final A2-101 construct and the 5' primer AAG CAT CAG AAT TGC CCC GCC ATG GTC GCA CTC GGC AGG GCC CCT CAC TTT were used to produce the A2-101 mutant.

The same approach as described for the construction of the A2-101 was carried out for the A6-101 mutant (p63 with deletion of amino acids 16 to 101), except for the 5' primer that was GCC CAT GCC CAT GCC CCT GCC CAA ACA AAG GGG CTC CAA GGG CCA CGG CCG CAG CTC GCG CAG CCT GCC CCT CAC TTT and used to produce the A6-101 AA mutant.

All other mutations were introduced by the overlap extension technique (Ho et al., 1989) using bp 774-791 of the p63 sequence and bp 170-193 of the Bluescript KS- vector as downstream and upstream primers. An appropriate partial complementary pair of oligonucleotides in which the desired mutation had been incorporated was chosen as internal primers. The final PCR products were digested with BamHI and used to replace the BamHI-BamHI fragment of pbKS-A6-101 (A6-101 introduced in EcoRI site of pbKS). Sequencing and further subcloning were as described above. Alternatively, final PCR products were directly subcloned into the Smal site of the pCEEC vector and sequenced.

For the construction of the A24-101 mutant (p63 with a deletion of amino acids 24 to 101), pbKS-p63 A6-101 was used as PCR template. The internal primers were GCC GCC AGC CCC TCG GAG AAG GGT GCC CGC AGG CTC GCG AGG GCC CCT CAC TTT for the downstream reaction.

Abbreviations used in this paper: ERGIC, ER-Golgi intermediate compartment; DPPIV, dipeptidylpeptidase IV; wt, wild type.

Materials and Methods

Materials

Enzymes used in molecular cloning were obtained from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega (Madison, WI). DME (4.5 g/l glucose) and RPMI-1640 medium were from Gibco BRL (Grand Island, NY); FCS from Hyclone (Logan, UT); and human plasma fibronectin from the New York Blood Center.

Oligonucleotides were synthesized with a solid phase synthesizer (380A; Applied Biosystems, Foster City, CA) by the Protein Chemistry Facility of Washington University.

Recombinant DNA

Materials

All basic DNA procedures were as described (Sambrook et al., 1989).

P63 Cytoplasmic Tail Mutants

The p63wt cDNA was as described previously (Schweizer et al., 1993b) and consisted of the 5' untranslated region, bp5-84, the 1803 nucleotide coding region and 1023 bp of the 3' non-coding sequence. The full-length cDNA was inserted into the EcoRI site of the Bluescript SK- vector (Stratagene, La Jolla, CA), respectively, with the initiation ATG facing the BamHI restriction site of the polylinker. The resulting constructs were designated pBSK-p63 or pbKS-p63, respectively. For transient expression in COS cells, the p63 insert was subcloned into the EcoRI site of the pCEEC vector (kindly provided by Dr. M. Spiess, Biozentrum, Basel, Switzerland) (Ellis et al., 1986) to give plasmid pCEEC-p63.

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All other mutations were introduced by the overlap extension technique (Ho et al., 1989) using bp 774-791 of the p63 sequence and bp 170-193 of the Bluescript KS- vector as downstream and upstream primers. An appropriate partial complementary pair of oligonucleotides in which the desired mutation had been incorporated was chosen as internal primers. The final PCR products were digested with BamHI and used to replace the BamHIBamHI fragment of pbKS-A6-101 (A6-101 introduced in EcoRI site of pbKS). Sequencing and further subcloning were as described above. Alternatively, final PCR products were directly subcloned into the Smal site of the pCEEC vector and sequenced.

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and GCG GCC ACC CTT CTC CGA GGG GCT GCC GCC GCT GCG GCC CCT GGC GCC CTG for the upstream reaction, respectively. The final plasmid was designated pECE-Δ24-101.

Alanine replacements of seven consecutive amino acids in the p63 cytoplasmic tail were encoded by GCT (for amino acids 5, 7, and 8) and GCC (for amino acids 5, 7, and 21) and GCT (for amino acids 8, 10, 11, and 22).

**P63-DPP IV Chimeras.** To construct fusion genes of p63 and dipetidylpeptidase IV (DPPIV), a cDNA for human DPPIV (Mismi et al., 1992; kindly provided by Dr. Y. Ikebana (Fukuoka University, Fukuoka, Japan)) was used. For the generation of the PD-P and Δ24-101 PDP chimeras (complete or Δ24-101 cytoplasmic tail of p63, transmembrane domain of p63, and luminal domain of DPPIV), the DPPIV cDNA was subcloned into a Bluescript KS−vector that has the Kpn I site in its polylinker deleted. A Kpn I site was then introduced between nucleotides 166 and 171 of the DPPIV sequence without altering the amino acid sequence. The resulting plasmid was digested with Sall and Kpn I to give a fragment including the complete luminal domain of DPPIV encoding the first 11 nucleotides (bp 160–170). The pECE-p63 or pECE-Δ24-101 plasmid, respectively, were digested with Sall and Not1, and the corresponding fragments that encode the complete or mutant (Δ24-101) p63 cytoplasmic tail plus the first 12 amino acids of the p63 transmembrane domain, were isolated. Two complementary oligonucleotides corresponding to nucleotides 441–465 of the p63 sequence followed by nucleotides 160–165 of the DPPIV sequence and a Kpn I site were sequenced and annealed. These components were assembled in a three-part ligation to give plasmids pECE-PDP or pECE-Δ24-101 PP, respectively.

All further chimeras were generated by PCR using the overlap extension technique (Horton et al., 1989). All chimeric constructs were precisely joined at the transitions between two domains. Final PCR products were treated as described above for the p63 cytoplasmic tail mutants, resulting in constructs pECE-DPP (cytoplasmic tail of DPPIV, transmembrane domain of p63, and luminal domain of p63), pECE-Δ24-101 DPP (Δ24-101 cytoplasmic tail of p63, transmembrane domain of DPPIV, and luminal domain of p63), and pECE Δ24-101 PDP (Δ24-101 cytoplasmic tail of p63, transmembrane domain of p63, and luminal domain of p63), respectively. The following templates were used for the PCR reactions: plasmids pBSK-p63 and pBSK-DPPIV (DPPIV cDNA subcloned as EcoRI fragment into the Bluescript KS−vector) to generate constructs DPP and DDP, and plasmids pBSK p63 Δ24-101 and pECE-DDP to construct the Δ24-101 PDP chimeras. Upstream and downstream flanking primers were the same as above. Appropriate pairs of partially complementary oligonucleotides which encoded the desired fusion were chosen as internal primers.

All mutants were verified by sequencing at the level of the final plasmid.

**Cell Culture and Transfection**

COS cells (African green monkey kidney cells, CRL 1650; American Type Culture Collection, Rockville, MD) were cultured in DME supplemented with 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, and fungizone at 37°C in a humidified 5% CO2 atmosphere.

For transient transfections COS cells were plated at 17,500-35,000 cells per well in an eight-well multichamber slide in which the wells had been coated for at least 20 min with 25 μl/microliter fibronectin in PBS, or at 104 cells per 60-mm plate. Subconfluent cells were washed once with TBS and each 60-mm dish was treated with 3 μg of DNA in 1.5 ml RPMI-1640 medium containing 10% Nusera, 50 μg/ml gentamicin, 100 μg/ml chloroquine, and 150 μg/ml DEAED-dextran (RNC plus DEAED-dextran) for 3.5 h. For eight-well multichamber slides 0.2 μg DNA in 100 μl RNC plus DEAED-dextran was used in each well. The DNA solution was then replaced with PBS containing 10% DMSO, cells were incubated for 2 min at room temperature, and washed with PBS before fresh medium was added. Cells were maintained at 37°C and analyzed 43-48 h after transfection.

**Antibodies**

Mouse mAb GI/296 against the p63 protein has previously been characterized (Schweizer et al., 1993a). For the detection of DPPIV mAb HBB 3/775 (Hauri et al., 1985) was used.

**Immunofluorescence Microscopy**

COS cells were grown in eight-well multichamber slides. The immunofluorescence procedure for permeabilized cells was that of Schweizer et al. (1988). In brief, formaldehyde-fixed and saponin-permeabilized cells were incubated with mAb GI/296 against p63 or mAb HBB 3/775 against DPPIV followed by goat anti-mouse FITC. To probe for surface expression of proteins, COS cells were cooled to 4°C for 20 min and kept on ice for the subsequent steps. The cells were washed once with PBS−0.2% BSA, and incubated with mAb GI/296 or mAb HBB 3/775 for 45 min. After six wash steps with PBS−0.2% BSA, the cells were fixed with 3% p-formaldehyde for 30 min on ice, followed by another 30 min at room temperature. Cell permeabilization and staining with goat anti-mouse FITC was as described (Schweizer et al., 1988). The specimens were examined with a Nikon fluorescence microscope and photographed with 1600 Fujichrome film.

**Immunoblotting**

After two washes with PBS, transfected COS cells of one 60-mm plate were scraped into 1 ml PBS and centrifuged for 10 min at 800 rpm (132 g).

The pellet was resuspended in 800 μl MINT buffer (20 mM MES, 30 mM Tris, 100 mM NaCl, 1.25 mM EDTA, and 1 mM EGTA) at various pHs containing 1% Triton X-100, 100 mM iodoacetamide, 40 μg/ml PMSF, and a 1:500 dilution of a protease inhibitor cocktail (5 mg/ml benzamidine and 1 mg/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethylsulfoxide, 60% ethanol) by passing it five times through a 25-gauge needle connected to a 1-ml syringe. After a 1-h solubilization step on ice, the cells were centrifuged for 60 min at 39000 rpm (100,000 g) in a Ti 50 rotor (Beckman Instruments Inc., Palo Alto, CA). The resulting supernatants were carefully harvested and proteins were precipitated by the method of Wessel and Flugge (1984). The precipitates were solubilized in Laemmli buffer (Laemmli, 1970). The pellets in the Ti 50 tubes following centrifugation were solubilized directly in sample buffer and sonicated. Proteins were separated on 8% SDS−polyacrylamide minigels (Bio Rad Laboratories, Richmond, CA) using the Laemmli (1970) system, and transferred to nitrocellulose membranes according to the method of Towbin et al. (1979). For the immunoreaction, the nitrocellulose sheet was blocked with 3% nonfat dry milk powder in PBS, incubated with mAb GI/296 against p63 (diluted 1:5000 in PBS−3% powdered milk) followed by a horseradish peroxidase conjugated anti-mouse secondary antibody (Amerham Corp.). For development the ECL detection system (Amersham Corp.) was used according to the manufacturer's directions.

**Affinity Purification of p63 Protein**

COS cells (10-12 60-mm plates per preparation) transfected with either p63wt or Δ2-101AA were washed twice with PBS, scraped into 1 ml PBS per plate and centrifuged for 10 min at 800 rpm (132 g). Each pellet was resuspended in 500 μl of buffer 2 (100 mM NaH2PO4, pH 8.0, 1% Triton X-100), containing 100 mM iodoacetamide, 40 μg/ml PMSF, and a 1:500 dilution of the above described protein inhibitor cocktail by passing it five times through a 25-gauge needle connected to a 1-ml syringe. After a 1-h solubilization step on ice, the cells were centrifuged for 60 min at 39000 rpm (100,000 g) in a Ti 50 rotor. p63 proteins were affinity purified from the resulting supernatants on a column of mAb GI/296 coupled to cyanogen bromide-activated Sepharose 4B. mAb GI/296 had been purified from culture supernatant by ammonium sulfate precipitation (to 50% saturation) followed by protein A-Sepharose chromatography prior to coupling according to the supplier's instructions. P63 proteins were eluted with 0.1 M glycine, pH 3.0, 0.05% Triton X-100, and immediately adjusted to pH 8.0 with 1 M Tris base. After analysis by SDS-PAGE, fractions that revealed a single band of appropriate size (Schweizer, A., J. Rohrer, and S. Kornfeld, unpublished data) were pooled and dialyzed against MNT buffer, pH 8.0, containing 0.05% Triton X-100. Protein was determined with the micro BCA protein assay (Pierce, Rockford, IL).

**pH Shift Experiment**

MNT buffer, pH 8.0, containing 1% Triton X-100 was added to 0.75 μg of purified p63wt or Δ2-101AA protein to a final volume of 700 μl before one of three incubations was carried out. The first sample was incubated for 40 min on ice. The second sample was brought to pH 5.8 by addition of a pretitrated volume of 1 N HCl and then incubated on ice for 40 min. The third sample was shifted to pH 5.8 and incubated on ice for 40 min; the pH was then returned to 8.0 by addition of a pretitrated volume of 1 N NaOH followed by another 40 min on ice. All samples were centrifuged for 60 min at 39000 rpm (100,000 g) in a Ti 50 rotor. The resulting supernatant and pellet fractions were further treated as described under immunoblotting.
Results

Expression of p63 cDNA in COS Cells

The endogenous p63 could be visualized in saponin-permeabilized COS cells by indirect immunofluorescence using a 1:20 dilution of mAb G1/296 which is specific for this protein. As shown in Fig. 1 a, the fluorescence pattern with its extended ER–Golgi intermediate membrane structure was very similar to that observed previously with Vero cells (Schweizer et al., 1993a). While ER-like, the pattern differs from that observed with ER markers in that it is less reticular and the outer nuclear membrane does not stain (see Fig. 2c for a typical ER pattern and Schweizer et al., 1993a). When the mAb G1/296 was tested at a dilution of 1:2,000, the p63 staining pattern was very faint (Fig. 1 b). Since this dilution of antibody gave a strong signal with p63 transfected COS cells (Fig. 2 a), it was possible to readily distinguish between endogenous and transfected p63. COS cells transfected with IRi3wt exhibited the typical extended membrane structure observed when endogenous p63 was stained (Fig. 2 a, left cell). In addition, some of the cells showed a large tubular network pattern that was not observed in the untransfected COS cells (Fig. 2 a, right cell) (Schweizer et al., 1993b). P63wt was never detected at the cell surface, even when the expression level was very high (Fig. 2 b).

The Cytoplasmic Domain of p63 Is Necessary for Retention

In our attempt to determine the features of p63 responsible for retention we initially concentrated on the cytoplasmic domain of the protein. The first construct to be prepared was a truncated form of p63 in which the amino-terminal residues 2-101 of the 106-amino acid-long p63 tail were deleted (A2-101, Fig. 3). Since p63 has a type II membrane orientation, the first NH2-terminal amino acid representing the initiator methionine could not be removed. When A2-101 was expressed in COS cells, it was clearly present on the plasma membrane in about half of the transfected cells (Fig. 2 d). Upon permeabilization, A2-101 was detected primarily in the rough ER as indicated by the reticular staining pattern along with labeling of the outer nuclear membrane (Fig. 2 c). Occasionally it was found in the Golgi region or at the cell surface (see insert of Fig. 2 c for an example of surface staining). In the construction of A2-101, two arginine residues became positioned near the extreme NH2-terminus due to the deletion of 100 amino acids within the cytoplasmic tail (Fig. 3). We therefore considered the possibility that these positively charged amino acids might serve as an ER-targeting signal, thereby impairing the normal transport of the protein (Jackson et al., 1990). These arginine residues (located in position -2 and -3 from the NH2 terminus of A2-101) were therefore changed to alanines (A2-101AA, Fig. 3) and the effect monitored by immunofluorescence as outlined above. A2-101AA showed strong cell surface expression over the entire transfected cell population (Fig. 2 f). Further, the staining pattern observed in permeabilized cells (Fig. 2 e) was typical for a cell surface protein including juxtanuclear Golgi and plasma membrane staining. Taken together, these data demonstrated that the cytoplasmic domain of p63 is necessary for correct intracellular localization.

The First 23 Amino Acid Residues of p63 Contain an Important Signal

To define more precisely the location of the retention information in the p63 cytoplasmic tail we made a construct (A16-101, Fig. 3) in which amino acids 2-15 of p63wt were added back to the A2-101 deletion mutant. When cells expressing this construct were analyzed by immunofluorescence, an internal staining pattern close to that of p63wt was observed except that no cells with the tubular network type pattern were found (Fig. 4 a). Labeling of nonpermeabilized COS cells with anti-p63 mAbs revealed that A16-101 is predomi-
Figure 2. The cytoplasmic tail of p63 is necessary for retention. P63wt (a and b), Δ2-101 (c and d), and Δ2-101AA (e and f) were expressed by transient transfection of COS cells and localized by indirect immunofluorescence of saponin-permeabilized (a, c, and e) and nonpermeabilized (b, d, and f) cells. For detection an mAb to p63 followed by goat anti-mouse FITC was used. In contrast to p63wt, constructs Δ2-101 and Δ2-101AA were observed at the plasma membrane. Bar, (a and b) 28 μm; (c-f) 42 μm.

nantly localized intracellularly (Fig. 4 b); an occasional cell showed weak staining of the plasma membrane (Fig. 4 b, inset). When the NH₂-terminal portion of p63 was extended to include the 23 NH₂-terminal residues of the p63wt sequence (Δ24-101, Fig. 3), the resultant protein behaved like p63wt when analyzed by indirect immunofluorescence after transfection (Fig. 4, c and d). The mutant protein was completely retained inside the cell (Fig. 4 d). In addition, the Δ24-101 staining in permeabilized cells was indistinguishable from that of p63wt (Fig. 4 c), including cells that exhibit the striking tubular network that is typically found in p63wt-transfected cells (Fig. 4 c, inset). We conclude from these results that the NH₂-terminal 23 amino acid residues of the p63 cytoplasmic domain are required for proper intracellular localization. Deletion of amino acids 24–101 of the p63 tail, on the other hand, had no detectable effect on the distribution of p63 in the cell.

To further define the sorting determinant that is present within amino acids 1–23 of p63, this region was subjected to extensive site-directed mutagenesis. First, a series of overlapping alanine stretches was created in the Δ24-101 mutant. As indicated in Fig. 5 A, amino acids 2–8 (Δ24-101, 2-8A), amino acids 6–12 (Δ24-101,6-12A), amino acids 9–15 (Δ24-101,9-15A), amino acids 13–19 (Δ24-101, 13-19A), or amino acids 17–23 (Δ24-101, 17-23A), respectively, were simultaneously changed to alanines using PCR mutagenesis. Surprisingly, these substitutions had no detectable effect on the localization of p63. Immunofluorescence of transfected nonpermeabilized cells showed that none of the mutants were transported to the plasma membrane (for an example see
These results clearly show that alanine substitutions have an effect when only amino acids 1-15 ($\Delta$16-101) instead of amino acids 1-23 ($\Delta$24-101) of the p63 cytoplasmic tail are present. One possible explanation for this finding could be the existence of a redundant signal with one of the components being located within residues 16-23.

When we carefully inspected the critical p63 sequence, a repetitive feature consisting of a positively charged amino acid followed by a glycine residue became apparent. The sequence contains an arginine-glycine combination at amino acid positions 7 and 8, and two pairs of lysine-glycine at positions 10 and 11, and 21 and 22, respectively. In addition,
Figure 5. Alanine scan of the cytoplasmic tail of (A) Δ24-101 and (B) Δ16-101. The amino acids of the cytoplasmic tail of Δ24-101 and Δ16-101 are shown (single letter code). Boxes represent the single transmembrane domain of p63. Bars indicate amino acids of the wt sequence substituted by alanines.

Figure 6. Immunofluorescence localization of selected constructs from the alanine scans shown in Fig. 5. COS cells were transfected with Δ24-101,6-12A (a and b) and Δ16-101,6-12A (c and d) and labeled with mAb G1/296 against p63 followed by goat anti-mouse FITC with (a and c) or without (b and d) permeabilization. Bar, 42 μm.
there is a single lysine residue present at amino acid position 5. The distribution of these residues relative to the amino acids altered in the overlapping alanine substitution experiments was consistent with these residues serving as components of a redundant retention signal. To test this possibility, K², R¹, G⁰, K¹⁰, G¹¹, K¹⁴, and G²² in the Δ24-101 sequence were simultaneously changed to alanines (Δ24-101, K²R¹G⁰K¹⁰G¹¹K¹⁴G²²-A [MP1]; Fig. 7). Immunofluorescence analysis of this mutant expressed in COS cells revealed that it was highly expressed at the plasma membrane of non-permeabilized transfected cells (Fig. 8 b). Furthermore, in permeabilized cells transfected with MP1 the staining was also characteristic for a surface protein (Fig. 8 a). The phenotype of MP1 is therefore very similar to that of the tail-minus mutant Δ2-101AA.

We next tested whether alanine substitutions of only the positively charged amino acids (K², R¹, K¹⁰, and K¹⁴) (Δ24-101, K²R¹K¹⁰K¹⁴-A [MP2]; Fig. 7) or only the glycine residues (G⁰, G¹¹, and G²²) (Δ24-101,G⁰G¹¹G²²-A [MP3]; Fig. 7) present in the K-RG-KG-KG element would also disrupt p63 localization. Both constructs behaved similarly to Δ24-101 and p63wt as indicated by the lack of cell surface staining (Fig. 8, d and f) and the typical internal pattern (Fig. 8, c and e). This demonstrated that changing positive charges or glycines alone does not affect retention of p63.

An additional construct was created that has the same mutation as MP1 except for amino acid position 5 where the original lysine was retained (Δ24-101, R⁰G⁰K¹⁰G¹¹K¹⁴G²²-A [MP4]; Fig. 7). Following transient transfection and immunofluorescence microscopy, this mutant showed two different localizations. Some of the chimeric protein was consistently detected on the cell surface (Fig. 8 h) while the remaining protein was predominantly found in the ER (Fig. 8 g). The latter finding suggested that the introduced changes might partly impair proper folding of the mutant protein which may prevent its exit from the ER. The presence of MP4 at the cell surface shows that K² cannot restore correct localization of p63 in the absence of the three positively charged amino acid-glycine combinations.

Analysis of Chimeric Proteins between p63 and a Plasma Membrane Protein

To further analyze the role of the cytoplasmic tail of p63 in retention and to determine whether the transmembrane and lumenal domains of this protein contribute to proper intracellular localization, we substituted each of these domains with the corresponding domains of the cell surface protein human DPPIV (Fig. 9). The serine protease DPPIV is a type II integral membrane protein like p63 and is found on a variety of epithelial, endothelial and lymphocytic cell types (Hong and Doyle, 1987, 1990; Ogata et al., 1989).

We first analyzed a chimeric protein (DPP; DPPIV cytoplasmic, p63 transmembrane, p63 lumenal; Fig. 9) in which the cytoplasmic tail of DPPIV was fused to the transmembrane and lumenal domains of p63. COS cells were transfected with DPPIVwt or the chimeric construct and the localization of the proteins was examined by indirect immunofluorescence of permeabilized and nonpermeabilized cells using mAbs to a lumenal epitope of each protein. Nontransfected control cells did not react with the anti-DPPIV mAb (data not shown) consistent with evidence that COS cells have little or no endogenous activity of DPPIV (Ogata et al., 1992). As shown in Fig. 10, a and b DPPIVwt was located on the cell surface as expected. Bright cell surface staining was also observed for DPP (Fig. 10 d). However, unlike DPPIVwt a portion of DPP was also found in the ER (Fig. 10 c), indicating that the export of this chimeric protein out of the ER is impaired to some extent. Very rarely a cell showed a p63wt-related staining pattern. A similar result was obtained with a construct that only contained the luminal domain of p63 (DDP; DPPIV cytoplasmic; DPPIV transmembrane, p63 luminal; Fig. 9) except that most of the ER staining was now replaced by cell surface expression (data not shown). These results confirm that the p63 cytoplasmic tail is necessary for proper localization. In the absence of the p63 cytoplasmic tail, the luminal p63 domain alone or in combination with its transmembrane domain is not sufficient to achieve retention.

We next asked whether the luminal domain of p63 is dispensable for the correct targeting of the protein. To this end, a chimeric construct in which the luminal domain of p63 was replaced by that of DPPIV (PPD, p63 cytoplasmic, p63 transmembrane, DPPIV luminal; Fig. 9) was created. In a related construct we linked the cytoplasmic and transmembrane domains of Δ24-101 to the DPPIV luminal domain (Δ24-101PPD, p63 with deletion of amino acids 24–101 cyto-
Identification of cytoplasmic residues critical for correct localization of p63. Constructs MP1 (a and b), MP2 (c and d), MP3 (e and f), and MP4 (g and h) were expressed in COS cells and localized by indirect immunofluorescence of permeabilized (a, c, e, and g) and nonpermeabilized (b, d, f, and h) cells. For detection an anti-p63 mAb and goat anti-mouse FITC were used. MP1 and MP4, but not MP2 and MP3 were found at the cell surface. Bar, 42 μm.

Figure 8. Identification of cytoplasmic residues critical for correct localization of p63. Constructs MP1 (a and b), MP2 (c and d), MP3 (e and f), and MP4 (g and h) were expressed in COS cells and localized by indirect immunofluorescence of permeabilized (a, c, e, and g) and nonpermeabilized (b, d, f, and h) cells. For detection an anti-p63 mAb and goat anti-mouse FITC were used. MP1 and MP4, but not MP2 and MP3 were found at the cell surface. Bar, 42 μm.
plasmic, p63 transmembrane, DPPIV lumenal; Fig. 9). When the subcellular localization of the chimeric proteins was analyzed in transfected COS cells, Δ24-101PPD showed the same distribution as DPPIVwt. There was strong labeling of the cell surface (Fig. 10 f), but no internal staining with a p63wt pattern (Fig. 10 e). The same was true for the PPD chimera (Fig. 10, g and h) except that the internal staining pattern included more ER staining in addition to the Golgi and cell surface staining predominantly found for DPPIVwt and Δ24-101PPD. These data demonstrate that the presence of the lumenal domain of the p63 molecule is essential for the correct localization of the protein.

The results with the PPD and Δ24-101PPD constructs together with the mutational analysis of the p63 cytoplasmic tail establish that the transmembrane domain of p63 is not sufficient for its localization. To determine whether this domain is a necessary component, we created an additional chimera that combines a p63 cytoplasmic tail that has amino acids 24–101 deleted with the transmembrane domain of DPPIV and the lumenal part of p63 (Δ24-101PDP; p63 with deletion of amino acids 24–101 cytoplasmic, DPPIV transmembrane, p63 lumenal; Fig. 9). Immunofluorescence microscopy of transfected cells showed that the internal distribution of Δ24-101PDP is very similar to p63wt (Fig. 10 i). Unlike p63wt, however, some Δ24-101PDP molecules were detected on the plasma membrane in unpermeabilized cells (Fig. 10 j). The intensity of this cell surface staining was fainter than that of DPPIVwt or any of the other chimeric constructs. Thus, while the transmembrane domain contributes to the complete intracellular retention of p63, it appears to be less important for the overall localization than its cytoplasmic and lumenal counterparts.

**Oligomerization of p63 Correlates with Its Retention**

The retention behavior of p63 described so far suggested that the underlying molecular mechanism might be related to physical properties within the p63 protein itself. Two findings in particular supported this idea. First, overexpression of p63 did not saturate the retention mechanism. And second, the p63 localization could not be transferred to another protein. We therefore speculated that individual p63 molecules might interact with each other to form higher order structures. To test this hypothesis p63wt was analyzed by centrifugation. In this experiment COS cells transfected with p63wt were solubilized with Triton X-100 at different pHs (5.8, 6.3, 6.8, 7.4, and 8.0) and then subjected to centrifugation at 100,000 g for 1 h. The resulting supernatant and pellet fractions were then assayed for the presence of p63 by SDS-PAGE and immunoblotting with an anti-p63 mAb. As shown in Fig. 11 A there was a pH dependent shift of p63 from the soluble fraction to the pellet. At pH 8.0, p63 was equally distributed between the pellet and supernatant fractions, whereas increasing amounts of p63 were recovered in the
COS cells transfected with p63wt were solubilized with Triton X-100 at the indicated pHs and then separated by centrifugation at 100,000 g into supernatant (S) and pellet (P). Proteins of the two fractions were subjected to SDS-PAGE (8% gels) and immunoblotting with an anti-p63 mAb. The numbers at the left margin of the blot indicate known molecular mass in kilodalton. (B) Samples of affinity purified p63wt protein (lanes 1-6) or A2-101AA (lanes 7-10) in MNT pH 8.0–Triton X-100 were incubated for 40 min (I), adjusted to pH 5.8 and then incubated for 40 min (II), or shifted to pH 5.8 for 40 min, then returned to pH 8.0 and incubated for another 40 min (III). Separation into supernatant (S) and pellet (P) and further analysis was as described in A.

Figure 11. P63wt forms Triton X-100–insoluble oligomers. (A) COS cells transfected with p63wt were solubilized with Triton X-100 at the indicated pHs and then separated by centrifugation at 100,000 g into supernatant (S) and pellet (P). Proteins of the two fractions were subjected to SDS-PAGE (8% gels) and immunoblotting with an anti-p63 mAb. The numbers at the left margin of the blot indicate known molecular mass in kilodalton. (B) Samples of affinity purified p63wt protein (lanes 1-6) or A2-101AA (lanes 7-10) in MNT pH 8.0–Triton X-100 were incubated for 40 min (I), adjusted to pH 5.8 and then incubated for 40 min (II), or shifted to pH 5.8 for 40 min, then returned to pH 8.0 and incubated for another 40 min (III). Separation into supernatant (S) and pellet (P) and further analysis was as described in A.

pellet as the pH decreased. When the experiment was performed with nontransfected COS cells at pH 7.4, 94 ± 8% of the endogenous p63 was recovered in the pellet fraction. This demonstrates that p63 oligomerization occurs at physiologic protein concentrations, excluding the possibility that this property is a special feature of transfection and/or overexpression. Similar results were obtained with affinity-purified p63, as shown in Fig. 11 B (lanes 1–6). In this experiment, purified p63 kept at pH 8.0 remained completely soluble (lanes 1 and 2). When the pH was shifted to 5.8, about half of the proteins formed oligomers that sedimented at 100,000 g centrifugation (lanes 3 and 4). This oligomerization was completely reversed by restoring the pH to 8.0 (lanes 5 and 6). Taken together, these results indicate that p63 forms Triton X-100–insoluble oligomers, especially at neutral pH and below.

We next tested whether the ability of p63 to oligomerize correlated with retention and proper localization. To address this point, a selected set of p63 cytoplasmic tail mutants were expressed in COS cells and the transfected cells were then solubilized with Triton X-100 at pH 6.5 and analyzed as described above. The results of a typical experiment are shown in Fig. 12 A and the quantitation of multiple experiments is given in Fig. 12 B. P63wt which served as a control was exclusively found in the pellet fraction (97 ± 5%). Δ24-101, MP2, and MP3 which have intracellular distributions similar to p63wt were also recovered predominantly in the 100,000 g pellet (81 ± 9%, 78 ± 4%, and 77 ± 5%, respectively). In contrast, Δ2-101AA and MP1 which are not localized properly, were found mainly in the supernatant (striated dark bars) fraction, respectively, is plotted as the percentage of p63 present in both fractions (y-axis). Each value is the average of at least four independent experiments. The standard deviations were <8.5%.

Figure 12. Oligomerization of p63 correlates with its retention. (A) COS cells were transfected with wt or mutant p63, solubilized with Triton X-100 at pH 6.5 and further analyzed as described in Fig. 11. (P) pellet, (S) supernatant. The upper band seen in the pellet lanes of p63 mutants represents endogenous p63 that is exclusively found in the pellet fractions. (B) The immunoblots shown in A and those from additional experiments were quantitated by densitometric scanning. For each sample (x-axis) the amount of wt or mutant p63 detected in the supernatant (dotted light bars) and pellet (striated dark bars) fraction, respectively, is plotted as the percentage of p63 present in both fractions (y-axis). Each value is the average of at least four independent experiments. The standard deviations were <8.5%.

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In summary, the data show a strong correlation between retention of p63 and the formation of Triton X-100–insoluble complexes.

**Discussion**

Previous studies have established that p63 is stably retained between the rough ER and the Golgi apparatus (Schweizer et al., 1993a). In the present study, we have analyzed the contribution of the individual p63 domains to retention by creating chimeric constructs with the type II plasma membrane protein DPPIV. The role of the cytoplasmic tail was further analyzed by deletions and point mutations within the p63 protein itself. The appearance of mutant p63 proteins at the cell surface and the concomitant loss of the characteristic p63wt internal staining pattern were used as criteria for loss of proper retention. Using these approaches, we have identified the domains of p63 that determine retention and obtained data that are consistent with the notion that oligomerization of p63 serves as a major retention mechanism.

Table I presents a summary of all the constructs analyzed in this study. The striking characteristic of p63 retention is that no single topological domain is sufficient to specify retention. Rather, all three domains are required to achieve complete intracellular retention. The cytoplasmic and luminal domains of p63 are most important for proper localization with the transmembrane domain playing a lesser role in this process. These findings with p63 differ from those obtained with most other proteins of the early secretory pathway where one domain has been shown to be sufficient for retention. In the case of resident luminal ER proteins, the four-amino acid sequence KDE; at the carboxyl terminus serves as retrieval signal (Munro and Pelham, 1987; Pelham, 1989, 1990). Similarly, KXXX/KXXX elements at the carboxyl terminus of the cytoplasmic domains of type I transmembrane proteins function as retrieval signals (Nilsson et al., 1989; Jackson et al., 1990, 1993). When transplanted onto marker proteins, these sequences are sufficient to cause retention in the ER. The carboxyl-terminal cytoplasmic domain of TGN38 is also sufficient for localization of chimeric proteins to the TGN (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). Other Golgi proteins are localized by means of their transmembrane domains (V-ace-tylglucosaminyltransferase I (Burke et al., 1992; Tang et al., 1992), galactosyltransferase (Nilsson et al., 1991; Aoki et al., 1992; Russo et al., 1992), and the M glycoprotein of avian coronavirus (Swift and Machamer, 1991; Machamer et al., 1993) or in association with the cytoplasmic and luminal flanking sequences (a 2,6-sialyltransferase (Munro, 1991; Dahdol and Colley, 1993). The Ei glycoprotein of the mouse coronavirus is an exception among the proteins localized to the Golgi apparatus (Armstrong and Patel, 1991). While a short deletion in the cytoplasmic tail destroyed retention, the cytoplasmic segment alone was not sufficient for retention. Similar to the finding with p63, most of the sequence appeared to be required for proper localization.

Molecular dissection of the cytoplasmic tail of p63 identified the NH$_{2}$-terminal 23 amino acids as being necessary for retention whereas amino acids 24–100 were dispensable for this function. Within the NH$_{2}$-terminal sequence, substitution of three pairs of amino acids each consisting of a positively charged amino acid followed by a glycine residue (R$,G$, K$^{10}$, G$^{11}$, K$^{21}$, and G$^{22}$) by alanines (MP4) resulted in transport of the mutant protein to the plasma membrane, indicating the importance of the corresponding amino acids for correct localization of p63. Interestingly, changing either the positively charged residues or the glycines alone (MP2, MP3) did not affect retention of p63. Further, the elements required for retention appear to be at least partly redundant as revealed by the analysis of the overlapping alanine scan of Δ24-101, a similar scan of Δ16-101, and the cytoplasmic tail deletion mutants.

What is the role of the cytoplasmic tail in conferring proper intracellular retention of p63? A potential clue emerged when we analyzed the solubility of the various constructs in the nonionic detergent Triton X-100. When transfected cells were solubilized with Triton X-100 at pH 6.5 and then subjected to ultracentrifugation at 100,000 g, the proteins that were properly retained (p63wt, Δ24-101, MP2, and MP3) were predominantly found in the pellet fraction whereas the nonretained proteins (Δ2-101AA and MP1) were mostly recovered in the supernatant. Endogenous p63 from COS cells was exclusively found in the 100,000 g pellet, demonstrating that the property is not a function of transfection and/or overexpression. While the extent of Triton X-100–insolubility of p63wt increased with decreasing pH, most of the protein was insoluble at pH 7.4, which is similar to the pH of the cytoplasm. Since highly purified p63wt exhibited the same pH-dependent insolubility in Triton X-100, we conclude that this behavior is an intrinsic property of the protein. This does not exclude the possibility that p63 also interacts with other resi-

**Table I. Summary of Immunofluorescence Analysis of p63wt and Mutant Proteins**

| Construct | p63wt pattern | Surface staining |
|-----------|---------------|-----------------|
| p63wt     | yes           | no              |
| Δ2-101    | no            | yes             |
| Δ2-101AA  | no*           | yes             |
| Δ16-101   | yes*          | no              |
| Δ24-101   | yes           | no              |
| Δ24-101,2-8A | yes         | no              |
| Δ24-101,6-12A | yes        | no              |
| Δ24-101,9-15A | yes         | no              |
| Δ24-101,13-19A | yes*       | no              |
| Δ24-101,17-23A | yes*       | no              |
| Δ16-101,2-8A | no*         | yes             |
| Δ16-101,6-12A | no          | yes             |
| Δ16-101,9-15A | no*         | yes             |
| MP1       | no            | yes             |
| MP2       | yes           | no              |
| MP3       | no            | yes             |
| MP4       | no            | yes             |
| DPPIVwt   | no            | yes             |
| DPP       | no*           | yes             |
| DDP       | no*           | yes             |
| Δ24-101 PPD | no          | yes             |
| PPD       | no*           | yes             |
| Δ24-101 FDP | yes         | yes f           |

* Except no cells with tubular network pattern.
§ Occasionally cells showed weak staining of the plasma membrane.
~ Some cells showed a p63wt-related staining pattern.
† Very rarely a cell showed a p63wt-related staining pattern.
‡ The intensity of this cell surface staining was fainter than that of DPPIVwt or other chimeric constructs.
dent proteins of the compartment or with cytoplasmic elements, such as members of the cytoskeleton.

The striking correlation between proper intracellular localization and Triton X-100-insolubility suggests a retention mechanism for p63 that involves homooligomerization. The model predicts that upon arrival at their final destination, p63 molecules encounter an environment that leads to self-aggregation resulting in the formation of large oligomers. The specific conditions that might trigger the oligomerization of p63 are unknown, but could include differences in ion composition, lumenal pH or properties of the local membrane. This proposed retention mechanism is compatible with several additional findings. First, high levels of expression do not result in mislocalization of p63 to the plasma membrane, indicating that the p63 retention system is not saturable. If p63 interacted with a specific receptor, overexpression would likely lead to saturation of the retention machinery and the appearance of the protein at the cell surface. Second, as mentioned above, all three domains of p63 are necessary for complete retention, as would be expected if the molecule formed higher order structures. When Triton X-100-soluble p63 molecules were examined by sucrose gradient centrifugation or nonreducing SDS-PAGE, the majority of the molecules were present as noncovalently linked dimers (Schweizer et al., 1993a; Schweizer, A., J. Rohrer, and S. Kornfeld, unpublished data). Interestingly, this dimerization of p63 is independent on the cytoplasmic tail, in contrast to the formation of higher oligomeric complexes.

Taken together, the most straightforward interpretation of our results is as follows. The luminal domains of individual p63 molecules interact to form noncovalently linked dimers, with the transmembrane domains possibly having an accessory role. These dimers would represent the basic unit from which higher oligomeric structures are built. Upon reaching their final destination, larger complexes form, requiring interactions of the cytoplasmic tails as well as the luminal and transmembrane domains. The amino-terminal 23 residues of the cytoplasmic tail could either be directly involved in the interactions or, more likely, guarantee a conformation of the cytoplasmic tail that favors higher order assembly. The association of p63 into large complexes could prevent p63 molecules from entering budding vesicles and thereby provide a mechanism for retention. Alternatively, failure of the mutant p63 molecules to form the higher order complexes would result in leakage of the molecules out of the compartment with eventual delivery to the cell surface. This proposed model for the localization of p63 involves a retention rather than a retrieval mechanism, which is compatible with previous data suggesting that p63 is a stably anchored resident protein.

Oligomerization has also been suggested as a retention mechanism for proteins in the Golgi apparatus (Machamer 1991; Nilsson et al., 1991, 1993, 1994; Swift and Machamer, 1991). The only example where retention in this organelle has actually been correlated with oligomerization is a chimeric membrane protein in which the membrane-spanning domain of VSV G protein was replaced with the first membrane-spanning domain of the M glycoprotein of avian coronavirus (Weisz et al., 1993). However, in contrast to the chimera, oligomerization of the intact M glycoprotein could not be demonstrated. Recently, Nilsson et al. (1993, 1994) have proposed a model whereby Golgi enzymes form homo-dimers in which the luminal domains are bound together while the transmembrane domains interact with those of identical or related enzymes of the same Golgi cisternae to form hetero-oligomers. The cytoplasmic domains would be attached to an underlying matrix to stabilize larger arrays. Although similar in the overall architecture, this Golgi model differs from the model supported by the p63 data in several ways. In particular, the domains responsible for the formation of higher order structures (cytoplasmic tail versus transmembrane domain) and the kind of oligomers formed (homo- versus hetero-oligomers) are distinct. Taken together, oligomerization is likely to be a general mechanism that specifies location of proteins in different compartments of the secretory pathway. Oligomerization per se, however, seems to be achievable by different mechanisms and forms of interactions.

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