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A reversibly palmitoylated resident protein (p63) of an ER-Golgi intermediate compartment is related to a circulatory shock resuscitation protein

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SUMMARY

The recently identified 63 kDa membrane protein, p63, is a resident protein of a membrane network interposed in between rough ER and Golgi apparatus. To characterize p63 at the molecular level a 2.91 kb cDNA encoding p63 has been isolated from a human placenta λgt10 cDNA library. Sequence analysis of tryptic peptides prepared from isolated p63 confirmed the identity of the cloned gene. The translated amino acid sequence consists of 601 amino acids (65.8 kDa) with a single putative membrane-spanning region and a N-terminal cytoplasmic domain of 106 amino acids. The human p63 cDNA exhibits a high level of sequence identity to the pig hepatic cDNA 3AL (accession number M27092) whose expression is enhanced after resuscitation from circulatory shock. An additional remarkable feature of p63 is that it becomes reversibly palmitoylated when intracellular protein transport is blocked by the drug brefeldin A. Overexpression of p63 in COS cells led to the development of a striking tubular membrane network in the cytoplasm. This suggests that the protein may be determinant for the structure of the p63 compartment.

Key words: ER-Golgi intermediate compartment, p63 cDNA, palmitoylation

INTRODUCTION

Protein transport from the rough endoplasmic reticulum (ER) to the *cis*-side of the Golgi apparatus involves an ER-Golgi intermediate compartment (ERGIC) (Schweizer et al., 1990; Hauri and Schweizer, 1992). The ERGIC was originally defined by a novel 53 kDa non-glycosylated transmembrane marker protein (ERGIC-53) (Schweizer et al., 1988); it was cloned recently (Schindler, R., Zerial, M., Lottspeich, F. and Hauri, H.-P., unpublished data). In Vero cells, mAbs against ERGIC-53 label a tubulo-vesicular membrane system close to the *cis*-side of the Golgi apparatus that corresponds to the site at which ER-to-Golgi protein transport is blocked at 15°C (Schweizer et al., 1990). Isolation of the ERIC from Vero cells by a two-step subcellular fractionation procedure indicates that the ERGIC does not show typical features of its direct neighbor organelles (rough ER and *cis*-Golgi) with respect to a number of marker proteins (Schweizer et al., 1991), suggesting that it has unique properties and may be considered a functionally separate organelle. The ERGIC may correspond to the "budding compartment" of mouse coronavirus (Tooze et al., 1988) and to the "pre-Golgi vacuoles" of Semliki Forest virus-infected cells (Saraste and Kuismanen, 1984). The p58-positive pre-Golgi elements described by Saraste and Svensson (1991) may also be part of the ERGIC.

Immunofluorescence microscopy has revealed a temperature-dependence of the ERGIC-53 pattern (Schweizer et al., 1990; Lippincott-Schwartz et al., 1990). ERGIC-53 was found to be concentrated in close proximity to the Golgi apparatus upon shifting the temperature to 15°C. This raises the question of whether the ERGIC is a short-lived transport intermediate or a stable membrane structure. The latter notion was supported by the recent identification of a 63 kDa apparently non-glycosylated integral membrane protein, termed p63, that forms disulfide-linked dimers (Schweizer et al., 1993a). mAbs (monoclonal antibodies) against p63 recognized an extended reticular ER-Golgi intermediate membrane structure. While the distributions of ERGIC-53 and p63 overlapped by light and electron microscopy, the distribution of p63 was insensitive to

organelle perturbants such as low temperature and brefeldin A. Moreover, p63 was highly enriched in the isolated ERGIC fraction (Schweizer et al., 1993a). These findings suggest that the ERGIC may be larger than previously suggested by the ERGIC-53 analysis.

The features and functions of p63 are at present unknown. A 62 kDa peripheral membrane protein, p62, that has a location similar to p63 by cell fractionation was identified in CHO cells (Mundy and Warren, 1992) and reported to be reversibly palmitoylated. This acylation, which occurs by the post-translational attachment of the 16-carbon saturated fatty acid palmitate, has been observed in many viral and cellular proteins. In most cases acylation occurs via a thioester bond on a cysteine residue that is present in the cytoplasmic domain of the polypeptide near the membrane bilayer (Sefton and Buss, 1987). This modification can be either stable or reversible. Irreversible palmitoylation has been documented for different viral (Schmidt and Schlesinger, 1979; Schmidt et al., 1979; Schmidt, 1982) and other proteins (Crise and Rose, 1992) and takes place between the sites defined by 15°C (i.e. the intermediate compartment) and 1,2-mannosidase I activity (i.e. *cis*- to medial-Golgi) (Bonatti et al., 1989). On the other hand, proteins such as the mammalian transferrin receptor (Omary and Trowbridge, 1981), $p21^{N\text{-}ras}$ (Magee et al., 1987), a neuronal growth cone protein (GAP 43) (Skane and Viray, 1989), erythrocyte ankyrin (Staufenbiel, 1987), other erythrocyte cytoskeletal proteins (Staufenbiel, 1988) as well as the BC_3H1 protein p64 (James and Olson, 1989) have been demonstrated to undergo a reversible type of palmitoylation. p62 was reported to become reversibly acylated both during mitosis and in cells treated with intracellular transport inhibitors such as brefeldin A, monensin, carbonylcyanide *m*-chlorophenylhydrazine (CCCP) and aluminum fluoride. In light of other recent reports proposing a role for fatty acylation in intracellular transport (Glick and Rothman, 1987; Pfanner et al., 1989, 1990) the acylation and deacylation of p62 has been suggested to be important in vesicular transport.

In the present study, we sought to determine: first, whether a functional relationship exists between p63 and palmitoylated p62; and second, to isolate and characterize the cDNA encoding p63 as a prelude to more detailed structure-function studies.

MATERIALS AND METHODS

Immunoisolation of p63 and microsequencing

The 63 kDa protein was immunoprecipitated from Triton X-100-solubilized Caco-2 membranes using mAb G1/296 as described (Schweizer et al., 1988). The immunoprecipitate was subjected to SDS-PAGE and blotted onto a PVDF membrane with 25 mM Tris, 192 mM glycine, 0.5% SDS and 20% methanol as transfer buffer. After transfer, proteins were visualized with Coomassie blue R250 according to Matsudaira (1987). p63 was then digested from the membrane with 4 μg trypsin as described by Bauw et al. (1989). Tryptic peptides were separated on a VYDAC 218 TP 52 reverse-phase high-pressure liquid chromatography column according to Stone and Williams (1988). Sequence analysis of selected peptides was carried out on a 477 A pulse-liquid phase protein sequencer (Applied Biosystems Instruments) connected online to a 120A PTH-amino acid analyzer (Applied Biosystems Instruments). The peptide sequences were analyzed by using the programs of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

Table 1. Sequences of tryptic peptides of p63

Peptide	Amino acid position in p63 cDNA	Amino acid sequence
1	11 - 21	GGHGAASPSEK
2	147 - 161	QREELGQGLQGVEQK
3	162 - 177	VQSLQATFGTFESILR
4	247 - 260	SINDNIAIFTEVQK
5	291 - 294	EAVK
6	302 - 311	SREWDMEALR
* 7	312 - 326	STLQTMESDIYTEVR
8	327 - 332	ELVSLK
9	382 - 388	SDSHGPK
10	395 - 405	HSEAFEALQQK
*11	413 - 429	LQHVEDGVLSMQVASAR
12	430 - 440	QTESLESLLSK
13	454 - 472	LEGLGSSEADQDGLASTVR
14	473 - 489	SLGETQLVLYGDVEELK
*15	491 - 504	SVGELPSTVESLQK
16	532 - 539	LSSLDNLK

The amino acid sequences of all 16 peptides were found in the p63 cDNA (Fig. 2A). Peptides marked with an asterix were used to find the homology to the pig clone 3AL (see text).

cDNA cloning

Comparison of three internal tryptic fragments of p63 (Table 1, marked with an asterisk) with sequences deposited in the GenEMBL databank revealed a strong homology to a pig unidentified mRNA (cDNA clone 3AL, accession number M29072; Buchman et al., 1990). Approximately 800,000 plaques of a non-amplified, 1-4 kb size-fractionated human placenta λgt10 cDNA library (Morgan et al., 1987) were screened. The cDNA clone 3AL (see above) was ^{32}P-labeled using the random primed DNA labeling kit from Boehringer Mannheim. Duplicate filters (Schleicher and Schuell) were hybridized in 50% formamide, 1% SDS, 5 × SSC (750 mM NaCl, 75 mM sodium citrate), 5 × Denhardt's solution (0.1% each of Ficoll 400, bovine serum albumin and polyvinypyrrolidone), 100 μg/ml salmon sperm DNA with 2×10^6 cts/min radiolabeled probe (see above) at 42°C overnight followed by two washes at 42°C and four rinses at 55°C for 15 min each in 0.3 × SSC, 0.1% SDS. By this procedure 13 positive clones were identified and plaque purified. Lambda DNA was prepared, and the *Eco*RI inserts were subcloned into Bluescript SK vector (Stratagene) for restriction mapping and sequencing. In 8 positive clones cDNA fragments corresponding to p63 were found. One insert (14B) was ^{32}P-labeled as described above and used to screen the >4kb size-fractionated part of the above non-amplified human placenta λgt10 cDNA library (Morgan et al., 1987). Hybridization was performed as described above with 2.8×10^6 cts/min of radiolabeled probe. Wash conditions: twice at 42°C and four times at 60°C for 15 min each in 0.3 × SSC, 0.1% SDS. This screening resulted in the identification of 10 p63-related clones with the longest clone (135A) having an insert of 2.91 kb.

cDNA sequencing

Nucleotide sequences were determined with double-stranded templates by the dideoxy termination procedure (Sanger et al., 1977) using the Sequenase kit (US Biochemicals). Band compressions in the first 510 bp of the p63 sequence could only be resolved when deaza G/A^{T7} Sequencing™ mixes (Pharmacia P-L Biochemicals) were applied. The nucleotide sequence and deduced amino acid sequence of p63 were analyzed using the programs of

the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

Northern blot analysis

Poly(A)$^+$ mRNA was isolated from three 100 mm dishes of Caco-2 cells 7 days after confluence using the Quick Prep mRNA purification kit (Pharmacia P-L Biochemicals). Poly(A)$^+$ mRNA (3 μg) was denatured with formaldehyde, size-fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred to a Nytran filter (Schleicher and Schuell) by capillary blotting (Sambrook et al., 1989). The membrane was prehybridized for 6 h at 65°C in 1 M NaCl, 10% dextran sulfate, 0.5% SDS. Hybridization was then carried out for 16 h at 65°C in 1 M NaCl, 10% dextran sulfate, 0.5% SDS, 200 μg/ml of denatured salmon sperm DNA containing 4×10^7 cts/min of denatured ^{32}P-labeled probe (14B) that was labeled using the primer extension method with random primers (Feinberg and Vogelstein, 1983; Sambrook et al., 1989). The blot was washed once for 5 min in 2 × SSC (300 mM NaCl, 30 mM sodium citrate), 0.1% SDS at room temperature, twice for 10 min in 2 × SSC, 0.1% SDS at 65°C and once for 10 min in 0.2 × SSC, 0.1% SDS at 65°C. The blot was then analyzed by autoradiography.

Cell culture and transfection

Caco-2 cells were grown in Optilux Petri dishes (Becton Dickinson Co., Lincoln Park, NJ) in DMEM supplemented with 20% heat-inactivated fetal calf serum (FCS), 1% non-essential amino acids, 100 i.u./ml penicillin, 100 μg/ml streptomycin, 1 μg/ml fungizone, according to Pinto et al. (1983). COS and Vero cells were cultured in DMEM containing 100 i.u./ml penicillin, 100 μg/ml streptomycin, 1 μg/ml fungizone under standard tissue culture conditions using 10% and 5% FCS, respectively. COS cells were transiently transfected by the DEAE-dextran method (Cullen, 1987) with 400 ng of p63 cDNA in pECE vector (Ellis et al., 1986) per multichamber well or with 3.4 μg of the same DNA per 35 mm plate. The pECE vector was kindly provided by Dr. M. Spiess, University of Basel.

Antibodies

Mouse mAb G1/296 against the p63 protein has previously been characterized (Schweizer et al., 1993a).

Immunofluorescence microscopy

COS cells were grown in 8-well multichamber slides. The immunofluorescence procedure was that of Schweizer et al. (1988). In brief, formaldehyde-fixed and saponine-permeabilized cells were incubated with mAb G1/296 against p63 (diluted 1:2000 to clearly distinguish transfected from non-transfected cells), followed by goat anti-mouse FITC. The specimens were examined with a Reichert Polyvar fluorescence microscope.

Immunoblotting

After two washes with PBS, transfected, mock-transfected or non-transfected COS cells of two 35 mm plates were scraped into 1 ml PBS and centrifuged for 10 min at 800 revs/min (132 g_{av}). The pellet was resuspended in 300 μl PBS containing 40 μg/ml PMSF by passing it ten times through a 25 gauge needle connected to a 1 ml tuberculine syringe. Protein was determined with the Bio-Rad protein assay kit (Biorad Laboratories, München, FRG). Proteins were separated on a 10% SDS-polyacrylamide slab gel using the Laemmli system (Laemmli, 1970) and transferred to nitrocellulose membranes according to the method of Towbin et al. (1979). For the immunoreaction the nitrocellulose sheet was incubated with mAb G1/296 against p63 (1:1000 dilution) followed by rabbit anti-mouse antibody (1:500) and ^{125}I-Protein A in the presence of PBS/1% powdered milk (Hauri and Bucher, 1986).

Labeling with [^{3}H]palmitate

Vero cells of one 35 mm culture dish were washed twice with serum-free DMEM growth medium and labeled in 2 ml DMEM containing 5% FCS and 1 mCi of [^{3}H]palmitate (New England Nuclear, MA) for 90 min at 37°C in the presence or absence of 10 μg/ml brefeldin A. Control cells were labeled overnight with 100 μCi [^{35}S]methionine in 2 ml PBS/1% non-essential amino acids/20% dialyzed FCS at 37°C. After labeling p63 was immunoprecipitated from Triton X-100-solubilized cells by mAb G1/296 as described (Schweizer et al., 1988). Proteins were separated on 10% SDS-polyacrylamide gels (Laemmli, 1970) and visualized by fluorography using salicylate and Kodak XOmat AR films.

RESULTS

Analysis of p63 by microsequencing

We originally planned to screen a human cDNA library for p63 using a series of degenerate oligonucleotides coding for p63 amino acid sequences as probes. For this purpose p63 was immunoprecipitated from Triton X-100-solubilized Caco-2 cells, subjected to preparative SDS-PAGE, and blotted onto a PVDF membrane. This purification resulted in a protein that was pure enough for analysis by microsequencing. Edman degradation of the intact protein gave no conclusive results, indicating that the N terminus was blocked. The protein was therefore digested with trypsin, and three resulting fragments (marked with an asterisk in Table 1) were sequenced and initially analyzed using the GenEMBL databank. Unexpectedly, all three peptides were very similar to sequences deduced from a pig cDNA 3AL (accession number 27092; Buchman et al., 1990), a porcine gene of unknown function whose expression increases at least fivefold after resuscitation from cardiogenic shock (Buchman et al., 1990).

Isolation of cDNAs encoding human p63

The 1.26 kb insert of the 3AL cDNA was therefore used as a probe to screen a nonamplified, 1-4 kb size-fractionated human placenta λgt10 cDNA library (Morgan et al., 1987). A total of 13 positive clones were isolated and 8 clones could finally be correlated to the p63 cDNA. One clone, designated 14B (Fig. 1), had a 1.96 kb insert corresponding to nucleotides 1 to 1961 of the final p63 cDNA

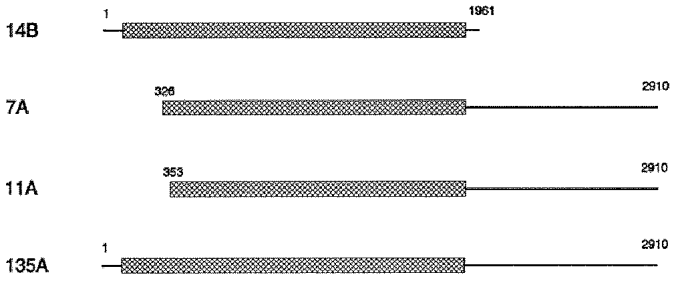

Fig. 1. Schematic representation of selected p63 cDNAs isolated from a human placenta λgt 10 library. The boxes indicate sequences of the coding region for the 601 amino acids of p63. Non-translated stretches are given as lines. The cDNAs 14B, 7A and 11A were obtained from the first screening (1-4 kb library) while clone 135A resulted from the second screening (> 4 kb library).

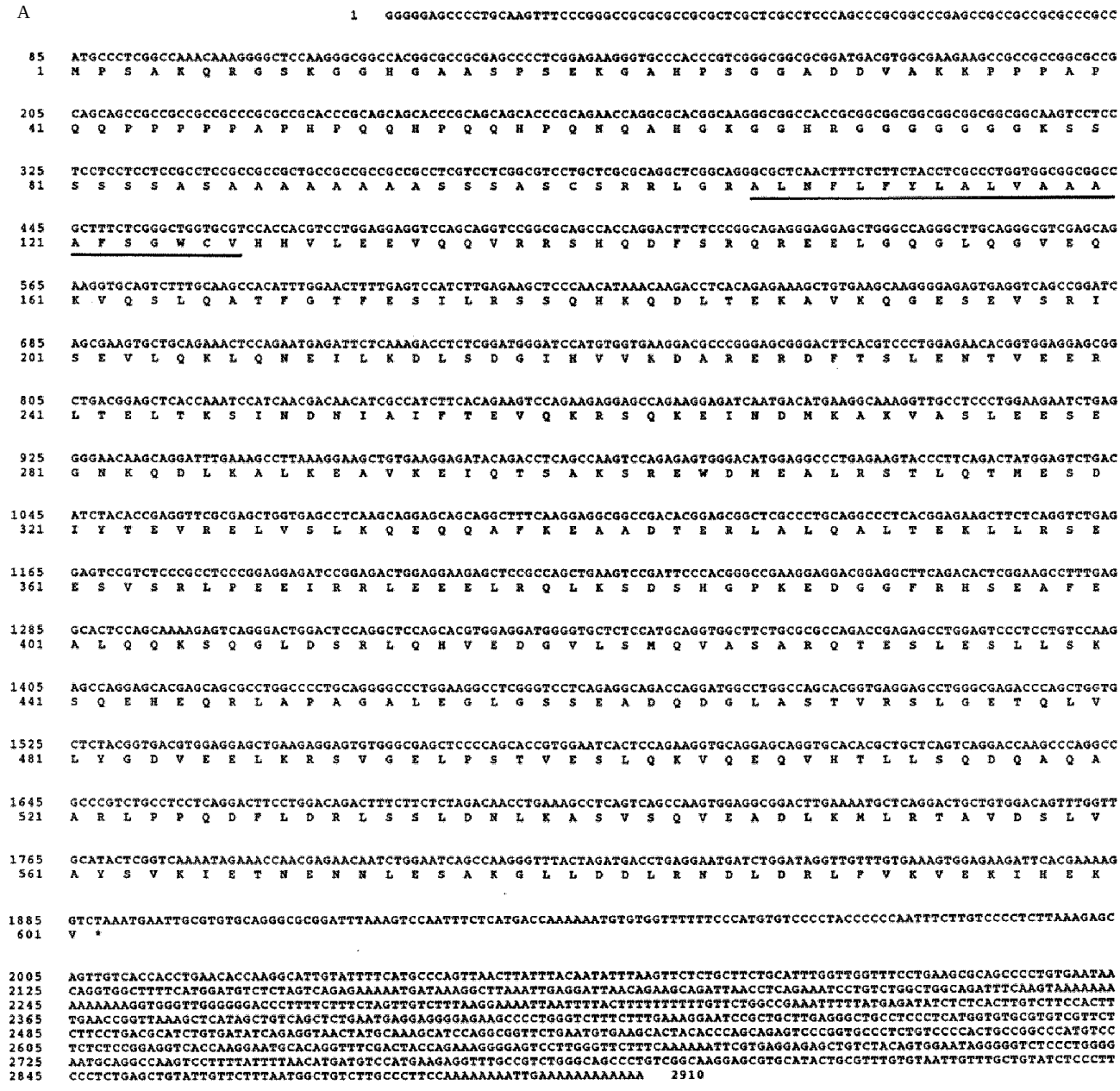

Fig. 2

(Fig. 2A). Clones 7A and 11A (Fig. 1) on the other hand showed 1636 and 1609 nucleotide overlaps, respectiveley, with the 3′ end of clone 14B, followed by 949 additional nucleotides.

To obtain a more complete cDNA the >4 kb size-fractionated part of the above human placenta cDNA library (Morgan et al., 1987) was rescreened using the ^{32}P-labeled 14B cDNA as probe. Of 800,000 recombinants 42 scored positive, 10 of which were selected for further studies. Most of these clones shared the 3′ end with the clones 7A and 11A but terminated prematurately between bp 168 and bp 443 of clone 14B. This region is extremely rich in G·C base pairs (CCG, GGC, TCC and GCC repeats) which might be the reason for the great number of incomplete cDNAs. The longest clone termed 135A, however, carried a 2.91 kb insert and comprised the entire sequence information of the above described partial clones. This 135A sequence contained a coding region of 1803 nucleotides as well as 84 bp of the 5′ noncoding sequence and 1023 bp of the 3′ noncoding sequence (Fig. 1; Fig. 2A). The 3′ untranslated region ends with a 12-base poly(A) tract but is not preceded by an upstream polyadenylation signal (AATAAA).

Characteristics of p63 cDNA: sequence analysis and comparision

The cDNA sequence of clone 135A predicts a protein of 601 amino acids (Fig. 2A) with a calculated molecular mass of 65.8 kDa, reasonably close to the 63 kDa determined by SDS-PAGE. The open reading frame included all three pep-

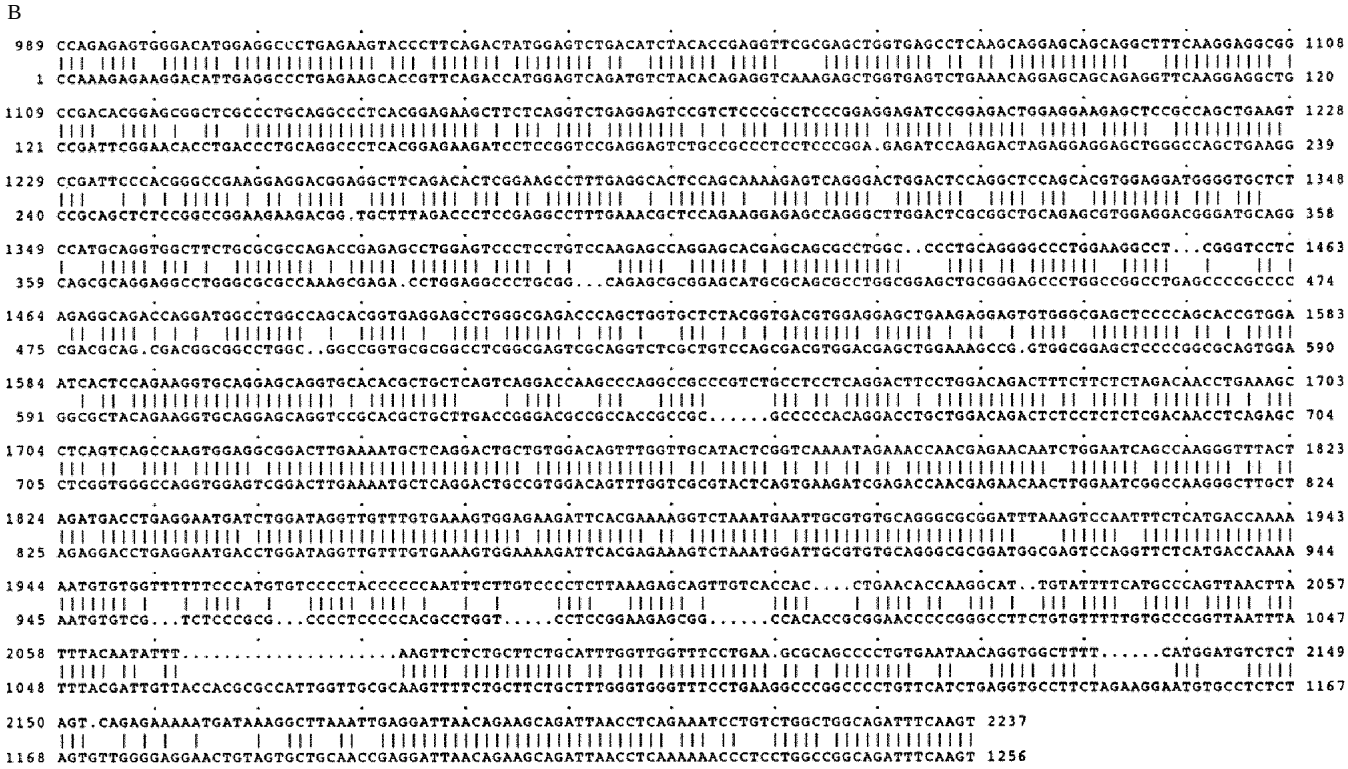

Fig. 2. (A) Nucleotide and deduced amino acid sequence of p63. Nucleotide positions are numbered on top of each lane; amino acid residues are numbered below. The one-letter amino acid code is used. The putative transmembrane sequence is underlined.
(B) Comparision of the human p63 (top) and the pig 3AL (bottom) sequence. The upper sequence represents bp 989 - 2237 of the human p63 cDNA (A). The bottom sequence represents the pig 3AL clone (accession number M29072). Base pairs are numbered in the right and left margins. Gaps were introduced to maximize the number of identical residues that are indicated by vertical bars. The computer best fit calculated 78.5 % identitiy.

tides derived from the initial sequence analysis of p63 (Table 1). Moreover, the amino acid sequences of 13 additional tryptic peptides were found (Table 1). The cDNA sequence surrounding the initiator codon (GCCATGC, bp 82-88) differs only at position +4 from the proposed consensus sequence for initiation by eukaryotic ribosomes (G/A CCATGG) (Kozak, 1986). The N terminus of the protein that follows the initiator methionine lacks the characterisitc features of a cleavable signal sequence (von Heijne, 1988). A hydropathy profile of the protein sequence according to Kyte and Doolittle (1982) (Fig.3) revealed a single potential membrane-spanning region of 21 amino acids (amino acids 107-127 in Fig. 2A; marked with an arrow in Fig. 3) that has the characteristics of an uncleaved internal signal peptide (von Heijne, 1988). p63 appears to be a type II transmembrane protein with an extracytoplasmic domain of 474 amino acids and a N-terminal cytoplasmic tail of 106 amino acids. This proposed transmembrane topology is supported by the previous protease protection experiments that suggested a 7 kDa cytosolic domain for p63 (Schweizer et al., 1993a). The most remarkable features of this cytosolic stretch are strings of consecutive identical amino acids (5 prolines, 7 glycines, 6 serines and 8 alanines, respectively), which account for the extreme G + C richness of this region. In agreement with the biochemical data (Schweizer et al., 1993a), the predicted amino acid sequence comprised no consensus site for N-linked glycosylation. There was also no other obvious motif present in the p63 cDNA sequence. The net charge of the protein at neutral pH is −18 and the isoelectric point was calculated to be 5.71.

Comparision with entries in the EMBL data bases revealed that the nucleotide sequence of the p63 cDNA is strikingly similar to that of the above described pig cDNA 3AL, whose transcript is related to cardiogenic shock resuscitation. In Fig. 2B the best alignment of the 3AL cDNA sequence and bp 989 - 2237 of the p63 gene is shown. 78.5% of the residues are identical over a stretch of 1249 nucleotides, which includes the entire 3AL region. The homology strongly suggests that human p63 is related to a porcine gene product transcriptionally induced during resuscitation from a clinically relevant model of circulatory shock.

Northern blot analysis of human intestinal mRNA with p63 cDNA

The mRNA size for p63 was determined by Northern blotting. Poly(A)$^+$ RNA prepared from the human intestinal cell line Caco-2 was hybridized under stringent conditions to a ^{32}P-labeled probe derived from the 14B cDNA. A single transcript of 5.0 kb was detected in Fig. 4. The size of the mRNA was unexpectedly large when compared to the 1.8 kb coding region of the p63 cDNA. This suggested longer stretches of nontranslated regions than observed in the

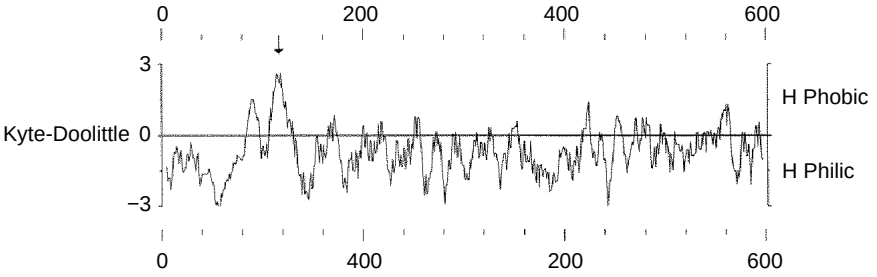

Fig. 3. Hydropathy plot of the predicited p63 amino acid sequence. Hydrophobicity was calculated according to the algorithm of Kyte and Doolittle (1982). Hydrophobic regions are above the horizontal line. The arrow indicates the 21 amino acid potential transmembrane domain marked in Fig. 2A.

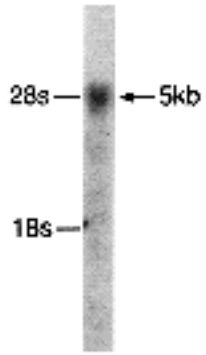

Fig. 4. Northern blot analysis of poly(A)$^+$ mRNA of Caco-2 cells using clone 14B as probe (see Materials and Methods).

135A clone that carries 84 bp of 5′ and 1023 bp of 3′ non-translated sequence.

Expression of p63 cDNA in COS cells

Transient expression of p63 in COS cells was performed using the pECE expression vector (Ellis et al., 1986). The p63/135A cDNA was subcloned into the *Eco*RI sites of the pECE vector and thus placed under the transcriptional control of the SV40 early promoter. The transcription unit is completed by a poly(A) addition signal and a poly(A) tract provided by SV40 sequences. This construct was used to transiently transfect subconfluent COS cells by the DEAE-dextran method (Cullen, 1987).

To verify that the isolated p63 cDNA encodes a protein indistinguishable from the endogenous p63, transfected cells were examined 48 h post-transfection by immunoblotting (Fig. 5). In this experiment equal amounts of protein (50-300 μg per lane) of homogenates from either transfected (lanes 1-4), mock-transfected (lanes 5-8) or non-transfected (lane 9) COS cells were subjected to SDS-PAGE, transferred to nitrocellulose sheets and probed with the anti-p63 mAb G1/296. This approach revealed a substantial overproduction of p63 after transfection compared to mock- and non-transfected cells. The immunoblot further demonstrated that the protein of transfected cells migrated as a single band with an apparent molecular mass of 63 kDa, coinciding exactly with the endogenous p63 protein.

We next examined the localization of the overexpressed p63 in COS cells 48 h post-transfection by indirect immunofluorescence microscopy (Fig. 6). The first antibody G1/296 against p63 was used at a high dilution (1:2000), which allowed clear distinction of transfected and non-transfected cells. The weak p63 staining pattern of non-transfected cells on the slides that were subjected to the transfection procedure was similar to the extended reticu-

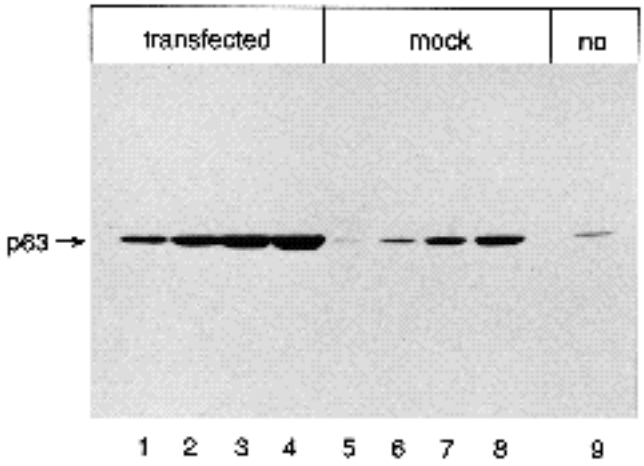

Fig. 5. Immunoblot analysis of p63-transfected, mock-transfected, and non-transfected COS cells with mAb G1/296. Homogenates of p63-transfected (lanes 1-4), mock-transfected (lanes 5-8) and non-transfected (lane 9) COS cells were prepared as described and equal protein amounts (50 μg (lanes1 and 5), 100 μg (lanes 2, 6 and 9), 200 μg (lanes 3 and 7), and 300 μg (lanes 4 and 8)) were subjected to SDS-PAGE (10% gel) and immunoblotting with mAb G1/296.

lar structure seen in COS cells that did not undergo the transfection procedure (not shown) or to the p63 pattern of Vero cells (Schweizer et al., 1993a). In about 40% of the cells a strong immunofluorescence signal was detected (Fig. 6) that was not identical to the endogenous p63 pattern (Schweizer et al., 1993a). In cells expressing p63 a large tubular network stained positive with the anti-p63 antibody. The immunoreactivity appeared either as dotted lines (Fig. 6A, B) or as continuous tubules (Fig. 6C,D). The two patterns did not seem to correlate strictly with expression levels. It is noteworthy that the characteristic immunofluorescence pattern is in general less reticular in appearance than that obtained by overexpressing KDEL-containing proteins in the rough ER of COS cells (Munro and Pelham, 1987). Furthermore, the p63 immunofluorescence pattern did not include labeling of the (outer) nuclear membrane, which would be characteristic of the rough ER.

p63 is palmitoylated in cells treated with brefeldin A

Recently, Mundy and Warren (1992) have reported the identification of a 62 kDa reversibly palmitoylated protein with a pre- and/or *cis*-Golgi localization. This protein is acylated in mitotic cells and in cells treated with intracel-

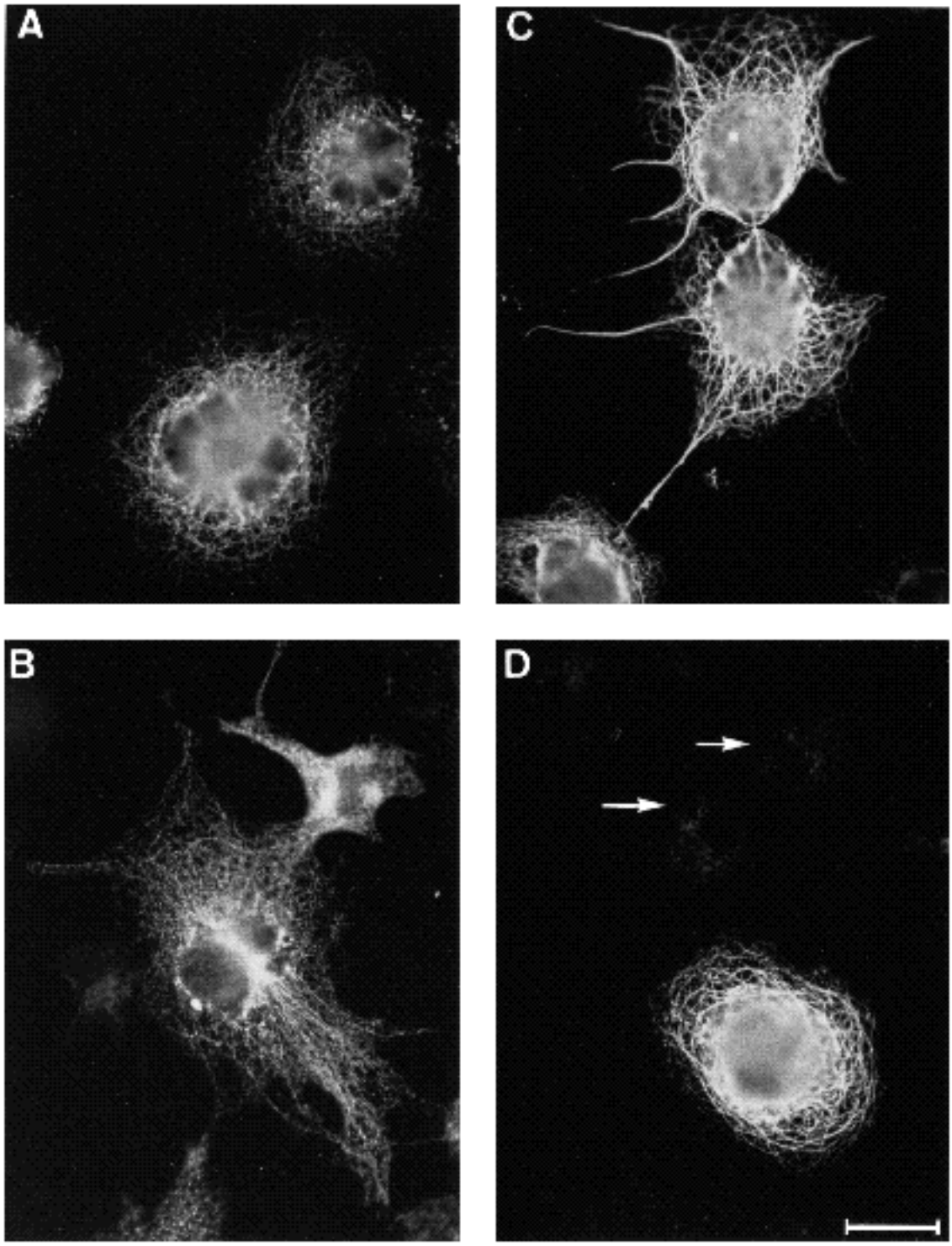

Fig. 6. Immunofluorescence analysis of p63-transfected COS cells with mAb G1/296. The cells were transfected with full-length p63 cDNA (clone 135A) using the DEAE-dextran method. The cells were fixed 48 h post-transfection and processed for indirect immunofluorescence. In some cells p63 appears as a dotted network (A,B), while in other cells p63 shows continuous tubules (C,D). The arrows indicate two cells expressing endogenous p63 only. Bar, 13.4 μm

lular transport inhibitors. Our p63 amino acid sequence reveals a cysteine (amino acid 100, see Fig. 2A) that is present in the cytoplasmic tail near the potential membrane-spanning domain. Cysteines close to the membrane bilayer have been found to undergo palmitylation via a thioester linkage (Sefton and Buss, 1987).

To test for palmitoylation, Vero cells were labeled with ^{3}H-palmitate for 90 min (Fig. 7, lane 2) or were incubated

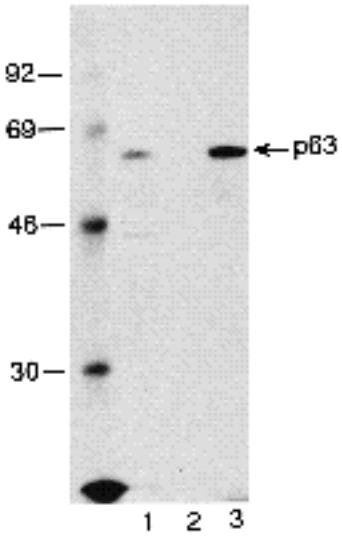

Fig. 7. p63 is palmitoylated after brefeldin A treatment. Vero cells were labeled with [^{3}H]palmitate for 90 min in the presence (lane 3) or absence (lane 2) of 10 μg/ml brefeldin A. Control cells (lane 1) were labeled with [^{35}S]methionine overnight. p63 was immunoprecipitated and subjected to SDS-PAGE (10% gel) followed by fluorography. The numbers at the left margin of the gel indicate known molecular mass in kilodaltons.

overnight with [^{35}S]methionine as control (Fig. 7, lane 1). The 63 kDa protein was immunoprecipitated and subjected to SDS-PAGE. As shown on the fluorogram in Fig. 7, p63 was labeled with [^{35}S]methionine (lane 1) but not with [^{3}H]palmitate (lane 2). When Vero cells were labeled with [^{3}H]palmitate in the presence of the fungal metabolite brefeldin A, label in a 63 kDa band was visible (Fig. 7, lane 3). After removal of brefeldin A, p63 lost the palmitate label with a half-time of about 30 min as determined by quantification of fluorograms (not shown). This is considerably faster than the half-life of the p63 protein as determined by [^{35}S]methionine labeling (Schweizer et al., 1993a). The result suggests that p63 is either not palmitoylated or only minimally palmitoylated in interphase cells while palmitoylation is inducible by brefeldin A in a reversible manner.

DISCUSSION

We recently identified a novel marker protein, p63, that overlaps in its distribution with the ERGIC defined by ERGIC-53 (Schweizer et al., 1993a). Herein, we describe the isolation and characterization of the human cDNA encoding p63. The identity of the cloned cDNA was confirmed by two different criteria. First, the sequences of 16 peptides that were obtained from a tryptic digest of the purified p63 protein were found in the open reading frame of the p63 cDNA (Table 1). Second, the cDNA, when transiently transfected into COS cells, produced a protein that is recognized by mAb G1/296 against p63. Its electrophoretic mobility is indistinguishable from that of the endogenous p63 protein. The latter finding also demonstrates that the first methionine codon (bp 85-87) represents the true initiator codon, although the cDNA sequence in Fig. 2A does not contain an upstream terminator codon. After transfection, the immunofluorescence pattern of p63 was somewhat different from that observed in non-transfected cells. In particular, some cells exhibited a striking tubular network. It appears that overexpression of p63 does not only fill an existing network but also changes its morphology. This raises the interesting question of whether p63 itself is a determinant for the topology of the p63 compartment.

Hydropathy analysis of the amino acid sequence deduced from the isolated cDNA suggests that p63 is a transmembrane protein spanning the lipid bilayer once. The putative transmembrane domain of 21 amino acids is localized near the N terminus of the protein. These findings are in accordance with previous biochemical data obtained for p63 (Schweizer et al., 1993a). The protein remained with the membrane fraction after carbonate extraction at pH 11.5. P63 was also not removed from membranes by urea concentrations up to 8 M (A. Schweizer and H.-P. Hauri, unpublished). Furthermore, a large portion of p63 was found to be protease-protected when postnuclear supernatants from Vero cells were treated with trypsin or proteinase K, except for a short segment of about 5 and 7 kDa, respectively. In addition, the N-terminal hydrophobic domain fulfils the criteria of an internal signal sequence, since it is flanked on the amino-terminal side by positively charged residues (von Heijne, 1988). Collectively, the data strongly suggest that p63 is a transmembrane protein with a large C-terminal domain on the extracytoplasmic side and a smaller N-terminal cytosolic tail.

The cDNA approach to analysis of ERGIC-53 showed not only that it was a type I transmembrane protein but also that it carried a double lysine motif in positions −3 and −4 from the C terminus (Schindler, R., Zerial, M., Lottspeich, F. and H.-P. Hauri, unpublished data). This double lysine motif is an organelle retention signal for some transmembrane proteins of the ER (Jackson et al., 1990; Wada et al., 1991; te Heesen et al., 1991; Shin et al., 1991). The presence of an ER retention motif in an ERGIC-associated protein may suggest either that this motif is a retrieval signal analogous to the KDEL retention signal described for organelle-retained but soluble proteins (Pelham, 1989; 1990, Peter et al., 1992; Schweizer et al., 1993b) or that the ER motif in ERGIC-53 is inactive. The p63 is a type II transmembrane protein which does not carry the double lysine retention signal. The mechanism by which p63 is retained in the ERGIC remains to be elucidated. Retention of three Golgi transmembrane proteins (all transmembrane Golgi proteins analyzed so far are type II) has been shown to depend primarily on the amino acid sequences embedded in the membrane with some additional contribution to retention by the sequences just adjacent to those membrane anchors (Nilsson et al., 1991; Munro, 1991; Swift and Machamer, 1991). It is possible that the ERGIC retention signal for p63 is similarly coded in the transmembrane domain; alternatively, a novel retention signal could be coded in the N-terminal cytosolic tail. Site-directed mutagenesis and expression studies should resolve this ambiguity.

Sequence analysis of human p63 cDNA showed a remarkably high homology to porcine 3AL, a gene transcriptionally induced in liver during resuscitation from circulatory shock. Circulatory shock resuscitation triggers the hepatic acute phase response, which includes marked increases in the secretion of procoagulants (such as fibrinogen) and antiproteases (such as α_1-antitrypsin); indeed we have previously shown that transcripts encoding these

proteins also accumulate in this model. If the sequence homology between porcine 3AL and human p63 predicts similar functions for the two gene products, then p63 could well be a hitherto unrecognized human stress response protein. Moreover, it suggests that expansion of the ERGIC could well be a crucial step in the hepatic acute phase response. We speculate that the marked increases in hepatic secretion of acute phase serum proteins depends, in part, on acceleration or expansion of the early secretory pathway.

Investigation of the acylation behavior of p63 has revealed that the protein is reversibly palmitoylated in the presence of brefeldin A but not or only little in the absence of the drug. Interestingly, Mundy and Warren (1992) have described a reversibly palmitoylated protein with a molecular mass of 62 kDa whose acylation is dramatically increased during mitosis and when intracellular transport is inhibited by brefeldin A. The increase was found to be specific for p62 and not accompanied by an increased palmitoylation of other proteins. Palmitoylation of p62 was also increased with monensin, CCCP, and aluminum fluoride, but the increase was less pronounced than with brefeldin A. For p63 a slight increase was seen under monensin (not shown) while no [^{3}H]palmitate labeling could be detected with CCCP or aluminum fluoride (not shown). Using subcellular fractionation p62 colocalized with p58 (Saraste and Svensson, 1991), indicating a pre- and/or *cis*-Golgi localization. Furthermore, p62 was reported to be tightly bound to the cytoplasmic side of membranes but it can be released by > 4 M urea. In contrast, p63 has been shown to be an integral membrane protein (Schweizer et al., 1992, and present paper) that remains membrane-associated even in the presence of 8 M urea (not shown). Mundy and Warren (1992) have studied only the palmitoylated form of p62 in their experiments, while we have looked at the total population of p63, which makes a comparison difficult. Whether the two proteins, p62 and p63, are indeed related to each other, as suggested by their similar acylation behaviour, remains to be shown. Since fatty acylation has been suggested to play a role in vesicle-mediated transport (Glick and Rothman, 1987) it will also be of interest to see if the reversible palmitoylation of p63 points to an involvement of the protein in intracellular protein transport in the early secretory pathway.

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