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Characterization of a novel 63 kDa membrane protein

Implications for the organization of the ER-to-Golgi pathway

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SUMMARY

Owing to the lack of appropriate markers the structural organization of the ER-to-Golgi pathway and the dynamics of its membrane elements have been elusive. To elucidate this organization we have taken a monoclonal antibody (mAb) approach. A mAb against a novel 63 kDa membrane protein (p63) was produced that identifies a large tubular network of smooth membranes in the cytoplasm of primate cells. The distribution of p63 overlaps with the ER-Golgi intermediate compartment, defined by a previously described 53 kDa marker protein (here termed ERGIC-53), as visualized by confocal laser scanning immunofluorescence microscopy and immunoelectron microscopy. The p63 compartment mediates protein transport from the ER to Golgi apparatus, as indicated by partial colocalization of p63 and vesicular stomatitis virus G protein in Vero cells cultured at 15°C. Low temperatures and brefeldin A had little effect on the cellular distribution of p63, suggesting that this novel marker is a stably anchored resident protein of these pre-Golgi membranes. p63 and ERGIC-53 were enriched to a similar degree by the same subcellular fractionation procedure. These findings demonstrate an unanticipated complexity of the ER-Golgi interface and suggest that the ER-Golgi intermediate compartment defined by ERGIC-53 may be part of a greater network of smooth membranes.

Key words: ER-Golgi intermediate compartment, 63 kDa protein, 53 kDa protein

INTRODUCTION

A major present challenge of cell biology is to relate the different steps of protein transport and retrieval to the subcellular structures of the cell. The pioneering studies of Jamieson and Palade (reviewed by Palade, 1975) on protein secretion in the acinar cells of the exocrine pancreas have led to the widely accepted view that newly synthesized exocytic proteins are packed into transport vesicles at the transitional elements of the rough endoplasmic reticulum (rough ER). The vesicles are targeted to the Golgi apparatus where they fuse with the *cis*-most Golgi element and thereby mediate the first step of exocytic interorganelle protein transport. Subsequent steps of protein transport through the secretory pathway are also envisoned to be governed by carrier vesicles (De Curtis and Simons, 1989; Wandinger-Ness et al. 1990; Rothman and Orci, 1990; Wilson et al., 1991).

The genetic analysis of secretory mutations in yeast (Hicke and Schekman, 1990) and the development of *in vitro* transport assays (Balch, 1989, 1990) has provided an important insight into the molecular machinery required for the vesicular transport from the ER to the Golgi apparatus. These studies have revealed a number of proteins that are involved in vesicle formation in a cooperative manner, such as Sec 12p, 13p, 16p, 23p (Kaiser and Schekman, 1990; Rexach and Schekman, 1991) and the ras-related GTPase Sar1p (Nakano and Muramatsu, 1989). At the restrictive temperature, yeast cells carrying mutations in these proteins accumulate exocytic proteins in the ER. In contrast, the gene products of *SEC17* (Kaiser and Schekman, 1990; which is homologous to a SNAP protein in higher eukaryotes: Clary et al., 1990), *SEC18* (Kaiser and Schekman, 1990; or its mammalian homologue NSF: Wilson et al., 1989, Beckers et al., 1989) and *SEC22* (Kaiser and Schekman, 1990) are involved in a later step of transport, i.e. vesicle fusion. Mutations in these genes lead to an accumulation of 50 nm vesicles, providing strong evidence for the vesicular nature of the ER-Golgi pathway in yeast. A vesicular ER-to-Golgi transport intermediate has indeed been isolated from yeast cells (Groesch et al., 1990; Rexach and Schekman, 1991). This intermediate was successfully used to reconstitute the in vitro transport of pro-alpha-factor to the Golgi apparatus. At least two ras-related GTPases, i.e. Ypt1p and its mammalian homologue Rab1 (Schmitt et al., 1988; Segev et al., 1988; Plutner et al., 1991) and ARF

(Stearns et al., 1990; Balch et al., 1992) are also required at this later step in the transport process from ER to Golgi and possibly in intra-Golgi vesicle movement as well (Serafini et al., 1991). Moreover, the ER to Golgi transport requires ATP and GTP at early and late stages, and calcium at only late stages (Beckers and Balch, 1989; Beckers et al., 1990; Rexach and Schekman, 1991). All these studies on vesicular transport are in accord with the Palade model of vesicular transport.

The process by which individual exocytic proteins are sorted from resident proteins along the ER to Golgi pathway has received considerable attention in the last few years (Rothman, 1987; Rothman and Orci, 1990; Pelham, 1989, 1991; Klausner, 1989; Mellman and Simons, 1992; Rothman and Orci, 1992). The prevailing current concept explaining protein transport is the bulk flow/retention model (Kelly, 1985; Pfeffer and Rothman, 1987). According to this model non-regulated exocytic protein transport does not depend on any specific signal but occurs by default (Wieland et al., 1987; Karrenbauer et al., 1990) while resident proteins are selectively retained in the ER by means of a retention signal. A retention signal was indeed found in a number of luminal ER proteins. They are retained by the C-terminal tetrapeptide signal KDEL or a closely related sequence (Munro and Pelham 1987; Pelham 1989, 1990) that was proposed to function in receptor-mediated retrieval from a post-ER salvage compartment (Warren, 1987; Pelham, 1988) rather than in directly attaching these proteins to the ER membrane. A putative KDEL receptor has been identified as a 26 kDa protein apparently localized in the Golgi apparatus (Semenza et al., 1990; Lewis and Pelham, 1990; Lewis et al., 1990; Lewis and Pelham, 1992).

It is at present unclear how the different steps of protein transport and retrieval relate to the subcellular structures of the cell. Morphological studies with higher eukaryotes indicate enormous complexity in the membrane structures between the ER and the Golgi apparatus. In addition to vesicles, both non-clathrin-coated and uncoated, thought to be involved in ER to Golgi transport (Merisko et al., 1987) there are smooth-surfaced tubular elements extending from both the rough ER and the *cis*-Golgi. In some cases these tubules appear to form direct connections between the two organelles (Lindsey and Ellisman, 1985a,b; Rambourg and Clermont, 1990; Tanaka and Fukudome, 1991). Tubulo-vesicular membrane elements interposed between the ER and the *cis*-Golgi element, termed the ER-Golgi intermediate compartment (ERGIC), have been shown to mediate ER-to-Golgi exocytic protein transport (Saraste and Kuismanen, 1984; Tooze et al., 1988; Schweizer et al., 1990). A membrane protein of 53 kDa (here termed ERGIC-53) was established as a marker for the ERGIC (Schweizer et al., 1988) and a mAb against ERGIC-53 labeled the site where the transport of exocytic proteins is arrested in cells cultured at 15°C (Schweizer et al., 1990). Furthermore, the GTPase rab2p largely colocalizes with ERGIC-53 at 37°C (Chavrier et al., 1990) and beta-COP, a major component of non-clathrin-coated vesicles, also associates with the ERGIC at 15°C (Duden et al., 1991b). Using ERGIC-53 as a marker, the ERGIC was successfully isolated from Vero cells and found to be different from its neighbor organelles, the rough ER and *cis*-Golgi, with respect to a number of marker proteins (Schweizer et al., 1991). A 58 kDa rat protein has similar subcellular distribution as ERGIC-53 in part (Saraste and Svensson, 1991; Hendricks et al., 1991). The relationship between the two proteins is unknown.

An unexplained observation concerns the temperature dependence of the ERGIC-53 pattern. Upon lowering the temperature to 15°C (or 16°C) ERGIC-53 concentrates preferentially near the Golgi apparatus (Lippincott-Schwartz et al., 1990; Schweizer et al., 1990). This could mean either that the ERGIC moves closer to the Golgi apparatus at 15°C or that ERGIC-53 itself undergoes temperature-dependent movement within a stable temperature-insensitive ERGIC. In the latter case it should be possible to find ERGIC markers that are not affected by temperature changes. To clarify this issue and to further elucidate the organization of the ER-to-Golgi exocytic pathway we have continued our search for organelle-specific markers using a mAb approach. Here we describe a novel 63 kDa protein which identifies an extended membranous network that appears to overlap with the ERGIC-53 compartment (Schweizer et al., 1988) and participates in exocytic protein transport. The results suggest that the ERGIC may be larger than previously anticipated on the basis of the ERGIC-53 marker.

MATERIALS AND METHODS

Materials

Reagents and supplies were obtained from the following sources. [^{35}S]methionine was isolated from bacteria grown in sulfur-limiting medium in the presence of [^{35}S]sulfate (New England Nuclear, Boston, MA/USA); Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l glucose), penicillin, streptomycin, fungizone, non-essential amino acids and dialyzed fetal calf serum (FCS) were from Gibco (Paiseley, Scotland); FCS from Readysystem (Kibbutz Beth Halmek, Israel); endoglycosidases H and F from Boeringer Mannheim (Mannheim, FRG); *O*-glycanase from Genzyme (Boston, MA/USA); neuraminidase from Calbiochem (La Jolla, CA, USA); phenylmethylsulfonyl fluoride, antipain, benzamidine, aprotinin, pepstatin and type III-O trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO, USA); proteinase K and SDS-PAGE reagents from Serva (Heidelberg, FRG); trypsin from Worthington Biochemical Cooperation (Freehold, NJ, USA); tetramethylrhodamine isothiocyanate (TRITC)-goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-gaot anti-mouse IgG from Cappel (Westchester, PA, USA); FITC-goat anti-mouse IgG1 and TRITC-goat anti-mouse IgG2a form Southern Biotechnology Associates, Inc. (Birmingham, UK); cell culture dishes from Falcon (Becton, Dickinson, Cockesville, MD, USA); and multichamber slides from Miles Laboratories (Naperville, IL, USA).

Cell culture

Vero cells (African green monkey kidney cells) were grown in Optilux Petri dishes (Becton Dickinson Co., Lincoln Park, NJ) in DMEM supplemented with 5% FCS, non-essential amino acids, 100 i.u./ml penicillin, 100 μg/ml streptomycin, 1 μg/ml fungizone under standard tissue culture conditions.

Antibodies

Mouse mAb G1/93 against ERGIC-53 protein has previously been characterized (Schweizer et al., 1988). A rabbit polyclonal anti-

body against ER proteins was generously provided by Dr. D. Louvard (Pasteur Institute Paris, France; Louvard et al., 1982). Mouse mAb G1/296 was obtained from the same fusion as mAb G1/93 (Schweizer et al., 1988) and is of the IgG2a subclass.

Conventional and laser scanning immunofluorescence microscopy

Vero cells were grown to 50% confluency in 8-well multichamber slides. The immunofluoresence procedure was as used by Schweizer et al. (1988). For double immunofluorescence, the formaldehyde-fixed, saponin-permeabilized cells were sequentially incubated with mAb G1/296, goat anti-mouse IgG2a-TRITC, mAb G1/93, and goat anti-mouse IgG1-FITC. The specimens were examined with either a Reichert Polyvar microscope or a Biorad MRC-500 confocal laser scanning microscope system attached to a Zeiss Axioplan microscope. Corrections of bleedthrough in double-labeling experiments were done according to the recommendations of the manufacturer and were tested in single-labeling experiments. Three types of organelle perturbation experiments were performed with the Vero cells: (I) incubation with 10 μg/ml brefeldin A for 2 h at 37°C; (II) incubation with 10 μg/ml nocodazole for 3 h at 37°C; (III) incubation at 15°C for 3 h.

Metabolic labeling with [^{35}S]methionine

Vero cells were grown in 30 mm dishes. At confluence the cells were rinsed with PBS, preincubated in PBS/1% nonessential amino acids/10% dialyzed FCS at 37°C for 10 min and pulsed for 3 h at 37°C with 200 μCi of [^{35}S]methionine in preincubation medium. Alternatively, the cells were continuously labeled at 37°C overnight with 100 μCi [^{35}S]methionine in preincubation medium supplemented with 10% complete Vero medium. For pulse-chase experiments the cells were rinsed with and chased in normal culture medium in the presence of 10 mM unlabeled methionine after a 15 min pulse with 250 μCi [^{35}S]methionine.

Immunoprecipitation, SDS-PAGE and fluorography

Antigens were immunoprecipitated from Triton X-100-solubilized cells as described (Schweizer et al., 1988). Proteins were separated on 10% SDS-polyacrylamide gels or 4% to 10% SDS-polyacrylamide gradient gels using the Laemmli (1970) system and visualized by fluorography using sodium salicylate and Kodak XOmat AR films.

Digestion with glycosidases, sodium carbonate extraction and protease protection experiments

Digestions with glycosidases were performed with the immunoprecipitated ^{35}S-labeled antigen as described previously (Matter et al., 1989). Membrane association was tested by the sodium carbonate procedure (Fujiki et al., 1982) as described (Gorr et al., 1988). For protease protection experiments the labeled cells were washed twice with PBS and homogenized in PBS by passing them 10 times through a 25G needle connected to a 1 ml tuberculin syringe. This homogenate was centrifuged for 10 min at 2000 revs/min (370 $\boldsymbol{g}_{av}$) in a SS34 rotor (Sorvall Instruments Division, Norwalk, CT). Aliquots of the resulting postnuclear supernatant were treated with trypsin or proteinase K as described (Schweizer et al., 1988).

Isolation of the ERGIC and quantification of p63 in subcellular fractions

The isolation procedure for the ERGIC-53 compartment was as described previously (Schweizer et al., 1991). p63 was quantified by a dot blot procedure based on the method of Hawkes et al. (1982) and was as follows. From the cell fractions to be assayed 4, 2 and 1 μl and 1 μl each of twofold serial dilutions in PBS were dotted onto a nitrocellulose filter. After air drying the samples were denatured by incubating the filter in 20% (v/v) methanol/7.5% (v/v) acetic acid. After 15 min the filters were rinsed three times with 0.05 M Tris, 0.2 M NaCl (pH 7.4) and incubated in the same buffer overnight. The filters were then blocked with 1% powdered milk in PBS for 30 min and sequentially incubated for 2 h with mAb G1/296 in blocking solution, a rabbit antibody to mouse immunoglobulins (1:500 diluted in blocking solution) and ^{125}I-Protein A (500,000 cts/min per/filter). Visualization and quantification of the labeled dots was as described by Schweizer et al. (1991).

Infection of Vero cells with vesicular stomatitis virus (VSV) and immunoelectron microscopy

Vero cells were infected with tsO45-VSV (Indiana serotype) as described earlier (Kreis 1986). The cells were kept at the non-permissive temperature (39.5°C) for 2.5 h followed by 2 h at 15°C in the presence of 10 μg/ml cycloheximide (Duden et al. 1991b). The cells were placed on ice, rinsed with PBS and the monolayer removed using ice-cold proteinase K (20 μg/ml) in PBS. To the cell suspension 16% paraformaldehyde in PBS was added to give a final concentration of 2% formaldehyde. This supension was centrifuged for 5 min at 1000 ***g***, the supernatant removed and 8% paraformaldehyde in 200 mM Hepes, pH 7.4, was carefully layered on the pellet. After 15 min the tube was centrifuged again at 13,000 ***g*** for 5 min and the pellet fixed overnight. Pieces of this pellet were mounted for cryosectioning and immunolabeling, as described earlier (Griffiths et al., 1984). For double labeling 1% glutaraldehyde was used to block between the two labeling steps, according to Slot et al. (1991).

RESULTS

mAb G1/296 identifies an extended cytoplasmic network

More than 300 hybridoma culture supernatants of a previously described cell fusion (Schweizer et al., 1988) were rescreened by immunofluorescence for the presence of antibodies against the ERGIC as defined by mAb G1/93 against ERGIC-53. The mAbs were produced against a Golgi fraction isolated from the human intestinal cell line Caco-2. No antibody was found with exactly the same pattern but one mAb, designated G1/296, showed an overlapping pattern with ERGIC-53. The corresponding hybridoma line was subcloned and concentrated antibody was prepared by ammonium sulfate precipitation or in the form of ascites. The specificity of both antibody preparations was identical in all the assays used in the study. By indirect immunofluorescence mAb G1/296 was found to label an extended structure in Vero cells (Fig. 1a). Although ER-like at first glance, the G1/296 pattern did not include staining of the (outer) nuclear membrane, which has characteristics of the rough ER. In contrast, a polyclonal antibody prepared against rough ER (Louvard et al., 1982) strongly and uniformely labeled the nuclear membrane and produced a reticular fluorescence pattern (Fig. 1c). The ERGIC-53 pattern was more focused on the

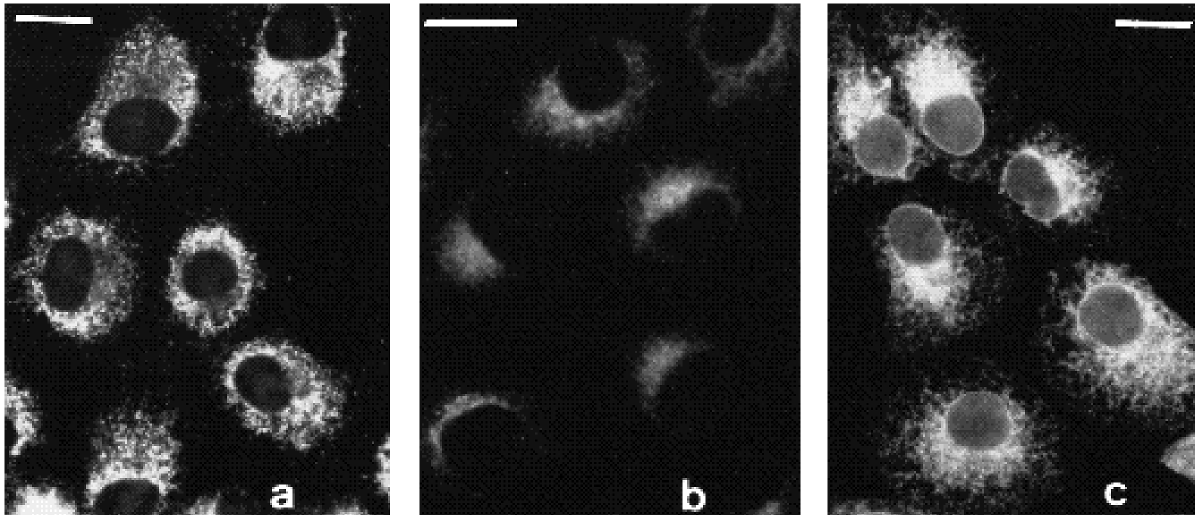

Fig. 1. Immunofluorescence localization of the G1/296 antigen (a), ERGIC-53 (b) and an ER marker (c) in Vero cells. Vero cells were fixed with paraformaldehyde and permeabilzed with saponin. The cells were labeled with mAb G1/296 or mAb G1/93 against ERGIC-53, followed by goat anti-mouse FITC or with a polyclonal anti-ER antibody (Louvard et al., 1982), followed by goat anti-rabbit TRITC. Bar, 20 μm.

Golgi area than that of G1/296 (Fig. 1b). This comparison showed that the G1/296 pattern was neither typical of ER defined by the Louvard antibody nor of the ERGIC defined by ERGIC-53. A similar pattern of G1/296 immunofluorescence was obtained in human liver cells (HepG2), human intestinal epithelial cells (Caco-2) and human MRC5 fibroblasts (not shown).

Identification and characterization of the antigen recognized by mAb G1/296

Before undertaking a detailed study on the compartment labeled by mAb G1/296, we decided to characterize the corresponding antigen biochemically. mAb G1/296 immunoprecipitated a single protein of 63 kDa (p63) from Vero cells metabolically labeled with [^{35}S]methionine (Fig. 2A) or from Caco-2 cells (not shown). Treatment of immunoprecipitated p63 with endoglycosidase H, endoglycosidase F or *O*-glycanase did not change its mobility on SDS-gels (Fig. 2A), suggesting that this protein is most likely not, or only minimally, glycosylated. p63 exhibited a relatively slow turnover as visualized by pulse-chase experiments (Fig. 2B). The antigen remained with the membrane fraction after carbonate extraction at pH 11.5 in the same way as the transmembrane protein ERGIC-53 that was used as a reference (Fig. 2C). We conclude that p63 is a membrane protein.

Treatment of a Vero cell homogenate with trypsin reduced the apparent molecular mass of p63 by 5 kDa. Proteinase K treatment resulted in a reduction of 7 kDa (Fig. 2D). Preincubation of the homogenate with Triton X-100 before protease treatment led to complete degradation of p63 in the case of proteinase K while partial resistance to trypsin was observed as indicated by the appearance of a 43 kDa proteolytic fragment of p63. The proteinase K data suggest that a large part of the membrane-bound p63 is protease-protected and that a segment of about 7 kDa may be exposed on the cytosolic side of the membrane.

The oligomeric state of p63 was assessed by SDS-PAGE under non-reducing conditions in the absence of dithiothreitol (Fig. 2E). Under these conditions p63 ran as two distinct bands at 63 kDa, 120 kDa and a poorly resolved smear higher than at 310 kDa. ERGIC-53 was used as a reference and gave the expected pattern (Schweizer et al., 1988). The result may indicate that p63 is a dimer in the intact cell. The smear in the high molecular mass region of the gel may represent aggregates or oligomers of p63.

Effect of organelle perturbants on the distribution of p63 and ERGIC-53

The structure of the ERGIC defined by ERGIC-53 depends on an intact microtubular network (Lippincott-Schwartz et al., 1990; Chavrier et al., 1990; Xiao and Storrie, 1991). To study if microtubule disruption affects the p63 and the ERGIC-53 patterns in the same way, Vero cells were treated with 10 μg/ml nocodazole for 3 h and subjected to double immunofluorescence analysis. A double immunofluorescence approach was feasible due to isotype differences between mAb G1/296 (an IgG 2a) and G1/93 against ERGIC-53 (an IgG1). Nocodazole treatment changed the reticular p63 pattern to “clouds” of immunofluorescence while the ERGIC-53 immunofluorescence was changed to a more punctate pattern of bright dots in addition to clouds (Fig. 3b). The clouds but not the dots co-labeled with mAb G1/296, suggesting that the two markers considerably overlap under these conditions (compare Fig. 3a and b).

Single immunofluorescence experiments showed that the pattern of p63 was little affected by brefeldin A (Fig. 4c) or by culturing the cells at 15°C (Fig. 4a). This is in contrast to ERGIC-53. Brefeldin A treatment induced dots of

immunofluorescence spread in the cytoplasm (Fig. 4d) while at 15°C ERGIC-53 concentrated in the Golgi area and in more peripheral dots of smaller size than those induced by brefeldin A (Fig. 4b). Collectively, these experiments showed that the distribution of p63 is less affected by organelle perturbants that affect protein trafficking than that of ERGIC-53, while both markers are equally sensitive to structural disruption (e.g. nocodazole).

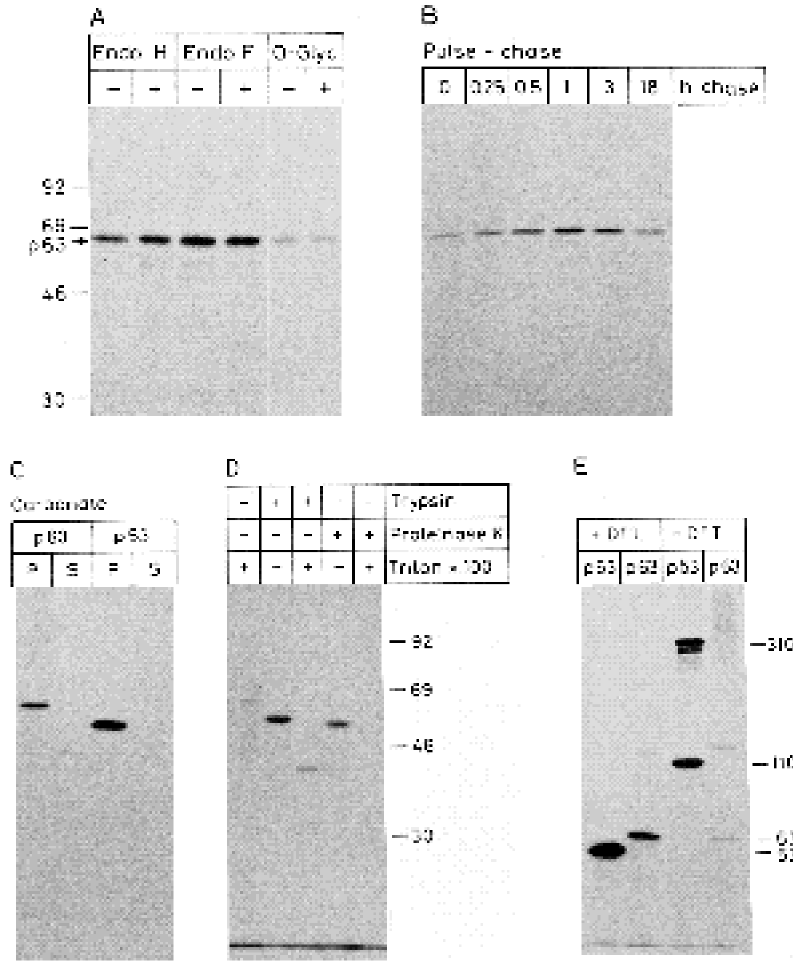

Fig. 2. (A) Identification of a 63 kDa protein in Vero cells by immunoprecipitation with mAb G1/296 and glycosidase digestion (fluorogram). Vero cells were labeled for 180 min with [^{35}S]methionine and the immunoisolated G1/296 antigen (p63) was subjected to Endo H, Endo F or *O*-glycanase treatment as indicated. Mock-treated controls without glycosidase treatment are shown in the minus lanes. Separation was on a 10% SDS-gel. The numbers at the left margin of the gel indicate [^{35}S]-marker proteins in kilodaltons. (B) The 63 kDa protein has a slow turnover. Vero cells were pulsed for 15 min with [^{35}S]methionine and chased with unlabeled methionine in excess for up to 18 h. p63 was precipitated with the G1/296 antibody and the immunoprecipitates were subjected to SDS-PAGE (10% gel) and fluorography. (C) Membrane association of the 63 kDa protein in Vero cells (fluorogram). Vero cells were metabolically labeled with [^{35}S]methionine and subjected to the carbonate extraction procedure as described under Materials and methods. (P) pellet, (S) supernatant. (D) Protease protection analysis of the 63 kDa protein in Vero cells (fluorogram). A postnuclear supernatant of labeled Vero cells was subjected to protease treatment as described under Materials and methods. Triton X-100, pretreatment of samples with 0.1% Triton X-100. (E) The oligomeric state of the 63 kDa protein. Triton X-100 extracts of [^{35}S]methionine-labeled Vero cells were precipitated with the G1/296 antibody. The immunoprecipitates were separated on a 4% to 10% gradient gel under reducing (i.e. in the presence of DTT) or nonreducing (i.e. in the absence of DTT) conditions. The numbers at the right margin of the gel indicate known molecular masses in kilodaltons.

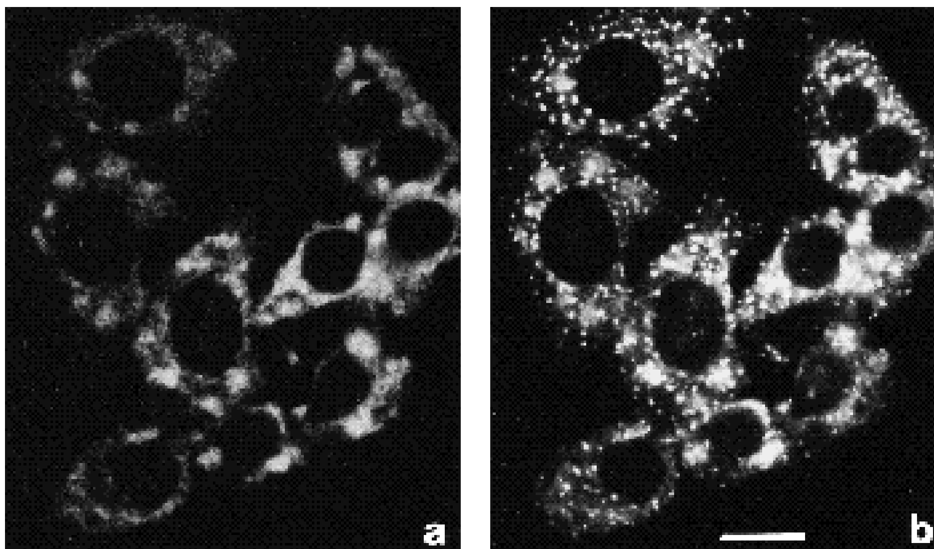

Fig. 3. Double-immunofluorescence localization of p63 (a) and ERGIC-53 (b) in Vero cells after nocodazol treatment. Vero cells were preincubated for 3 h with 10 μg/ml nocodazol, fixed with paraformaldehyde and permeabilized with saponin. The cells were labeled with mAb G1/296 against p63, followed by goat anti-mouse IgG2a TRITC and mAb G1/93 against p53, followed by goat anti-mouse IgG1 FITC. Bar, 20 μm.

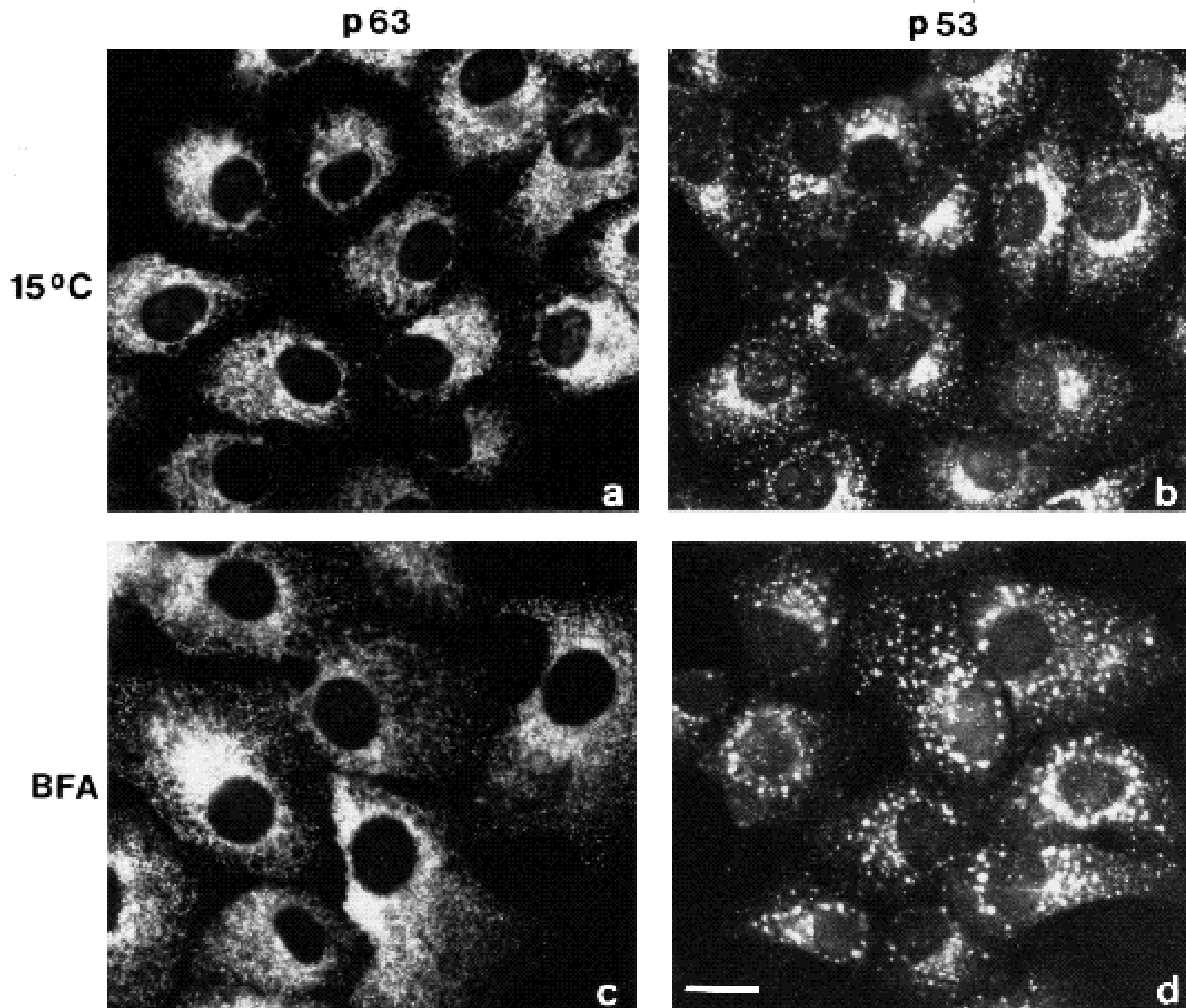

Fig. 4. Effect of low temperature (15°C) (a,b) and BFA (c,d) on the distribution of p63 (a,c) and ERGIC-53 (b,d). Vero cells were preincubated with either 10 μg/ml brefeldin A for 2 h or for 3 h at 15°C, fixed with paraformaldehyde and permeabilized with saponin. The cells were labeled with mAb G1/296 against p63 or G1/93 against ERGIC-53 followed by goat anti-mouse FITC. Bar, 10 μm.

Relationship between p63 and ERGIC-53 studied by confocal laser scanning immunofluorescence microscopy

The distribution and structural relationship between p63 and ERGIC-53 were examined at high resolution with a confocal laser scanning microscope by the acquisition of a series of optical sections from double immunofluorescence images (Fig. 5). Comparison of the FITC-labeled ERGIC-53 and and the TRITC-labeled p63 demonstrated that the relative distributions of the two proteins were essentially identical at all focal steps throughout the whole cytoplasm. However, ERGIC-53 was more concentrated in the Golgi area than p63. Superimposition of the projection of several optical sections confirmed this distribution and clearly revealed the presence of peripheral structures exclusively containing p63 (Fig. 6). It is noteworthy that neither antibody produced staining of the nuclear membrane.

Localization of p63 by immunoelectron microscopy

The distribution of p63 was studied by immunoelectron microscopy with mAb G1/296 on cryosections of Vero cells (Fig. 7). Specific labeling was found associated with smooth tubular profiles in the cytoplasm and with tubulo-vesicular structures close to the nucleus and the Golgi apparatus. Some of the labeled structures in the Golgi area displayed a distinct cytoplasmic coat that is characteristic of non-clathrin-coated vesicles. In previous studies identical profiles were labeled with antibodies against ERGIC-53 (Schweizer et al., 1990) or with antibodies against rab2p (Chavrier et al., 1990). Double immunoelectron microscopy with mAbs G1/296 and G1/93 showed co-labeling of some membrane profiles with both antibodies (Fig. 7B,C). Owing to the low labeling intensity it was not possible to quantify the extent of overlap but the labeling was sufficient to qualitatively demonstrate this point. We conclude, therefore,

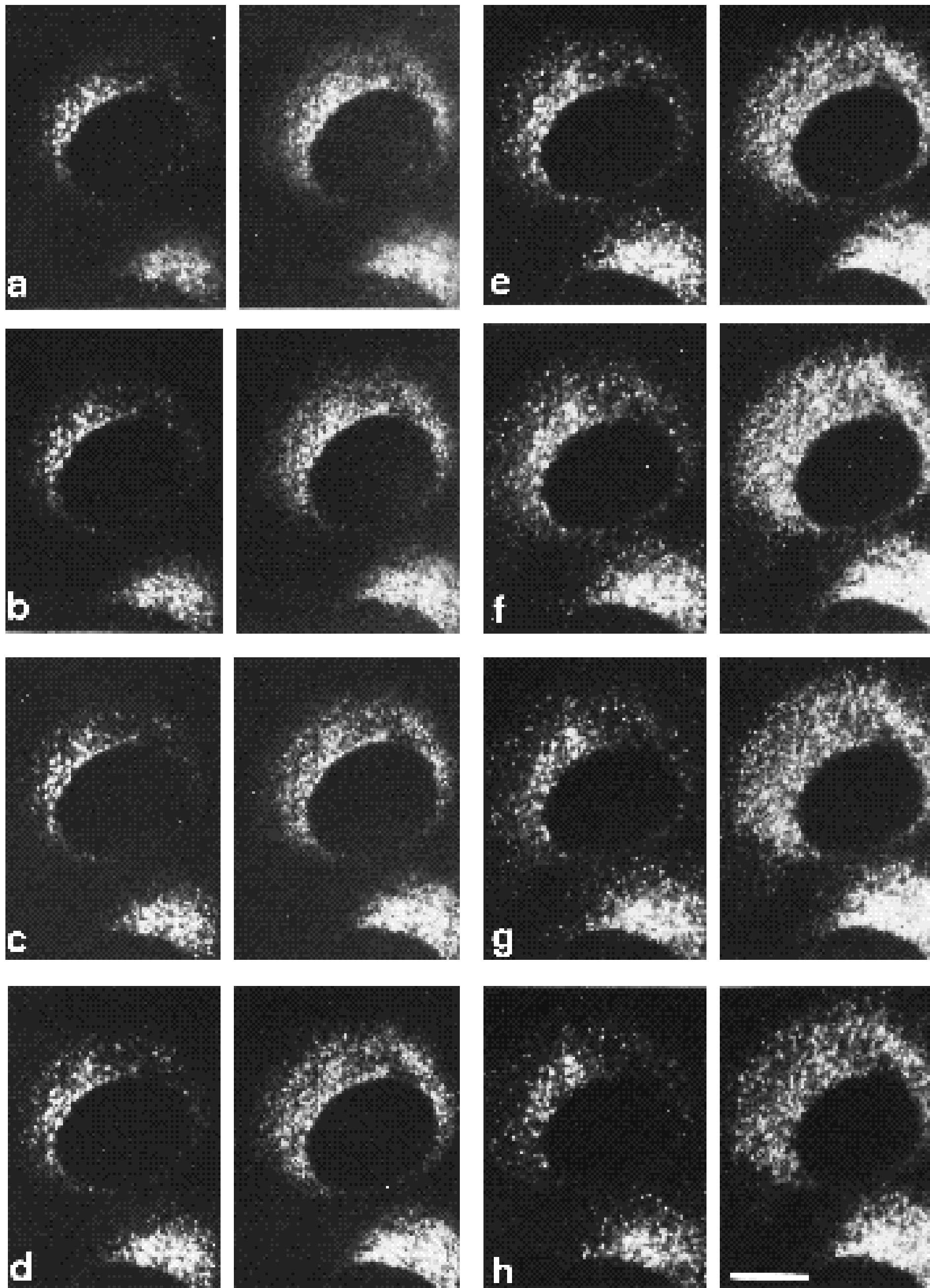

Fig. 5. Double immunofluorescence localization of ERGIC-53 and p63 in Vero cells analyzed by confocal laser scanning microscopy. A stack of 8 optical sections (0.4 μm apart) is shown. Simultaneous acquisition of images of FITC (ERGIC-53, left image) and TRITC (p63, right image) demonstrates that the structural relationship of the two proteins is identical through all levels (a - h) of the cytoplasm. Bar, 10 μm

that some of these membranes contain both markers. An unexplained finding is that some Golgi stacks occasionally show some labeling for p63 (Fig. 7) while the immunofluorescence experiments do not suggest labeling of the Golgi apparatus.

Previous studies have shown that newly synthesized vesicular stomatitis virus G protein colocalizes with ERGIC-53 in Vero cells cultured at 15°C (Schweizer et al., 1990). Since the p63 and the ERGIC-53 overlap it can be expected that the p63 compartment also participates in protein transport from the ER to the Golgi apparatus. Double immunoelectron microscopy in vesicular stomatis virus (VSV)-infected Vero cells with mAb G1/296 and a polyclonal antibody against VSV G protein provided direct evidence for this prediction (Fig. 8). In that experiment the VSV G protein of the ts045 mutant was arrested in the ER at 39.5°C and subsequently released into the 15°C compartment in the presence of cycloheximide. Four different sections in Fig. 8 document the accumulation of VSV G protein in tubulo-vesicular structures that are characteristic of ERGIC in Vero cells cultured at 15°C (Schweizer et al., 1990; Duden et al., 1991b). Some of the profiles co-label with the mAb aganist p63. Although low in general, the labeling for p63 was specific. The results suggest to us that the VSV G protein has access to the p63 compartment.

p63 is highly enriched in the isolated ERGIC

In a previous study we have established a subcellular frac-

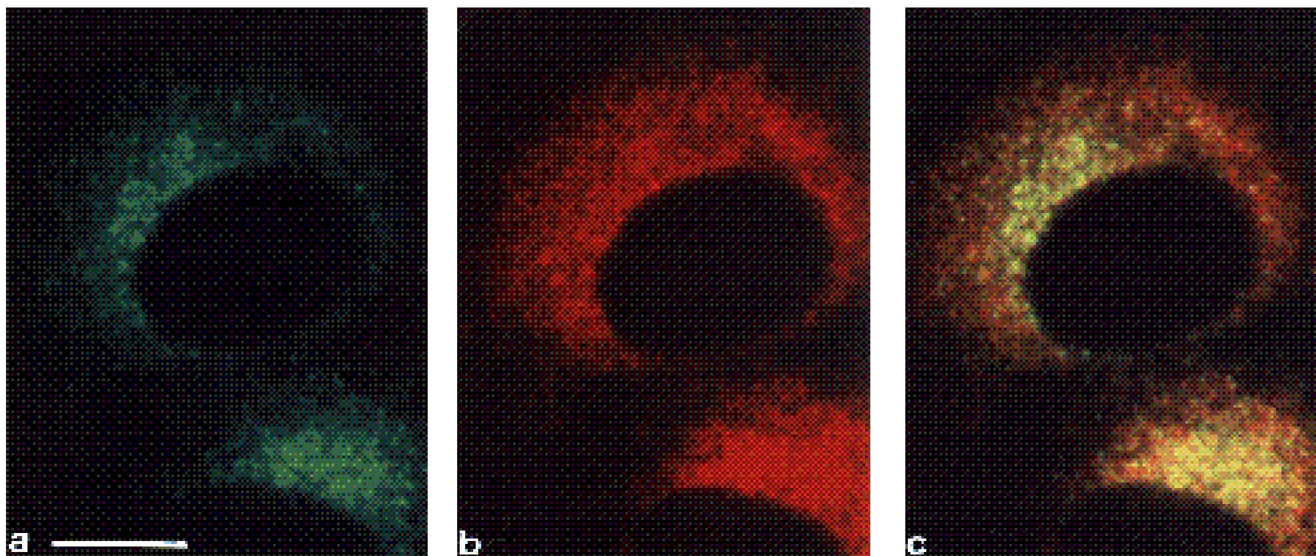

Fig. 6. Linear projection of the confocal images shown in Fig. 5 demonstrates that ERGIC-53 (a) is associated with coarser elements, which are predominantly localized in the Golgi region, whereas p63 (b) has a finer appearance and a more perinuclear distribution. Superimposition of the two images by image processing (c) reveals the presence of cytoplasmic peripheral elements which are solely labeled with anti-p63 (red), whereas the more central granules appear to be double stained (yellow). Bar, 10 μm.

tionation method for the purification of the ERGIC from Vero cells using ERGIC-53 as a marker protein (Schweizer et al., 1991). In essence this method is a two-step procedure in which a postnuclear supernatant is fractionated on a continuous Percoll gradient followed by a discontinuous Metrizamide gradient. P53 is 41-fold enriched in the final F3 fraction. Since p63 and ERGIC-53 overlap, the p63 protein should also be enriched in the isolated ERGIC. This was indeed the case. A dot blot procedure was developed to quantify p63 in the homogenate and in the F3 fraction (see Materials and Methods). This assay revealed a 65 ± 20 (n = 5) relative enrichment with a 36% yield of p63 in the F3 fraction versus the homogenate, indicating that both markers can be enriched to high purity with one and the same subcellular fractionation method. It is important to note that rough ER markers such as ribophorins I and II, PDI, BiP and signal sequence receptor (Schweizer, A. and Hauri, H.-P, unpublished data) are maximally 2.8-fold enriched in the F3 fraction (Schweizer et al., 1991). This low contamination clearly establishes that the p63-containing membranes we have isolated are not of rough ER origin.

Doluble immunoelectron microscopy with ultrathin cryosections of a F3 fraction confirmed the codistribution of p53 and p63. Some of the membranes in this fraction showed immunoreaction with antibodies against both markers (not shown).

DISCUSSION

In this study a mAb library produced against an isolated Golgi fraction of the human intestinal cell line Caco-2 (Schweizer et al., 1988) was screened for anti-ERGIC antibodies with the overall goal of elucidating the organization of the ER-to-Golgi pathway of exocytosis. This analysis resulted in the identification of p63, evidently a non-glycosylated membrane protein.

p63 is a resident protein of stable ER-Golgi intermediate membranes

Previously a tubulo-vesicular ER-Golgi intermediate compartment (termed ERGIC) was identified in Vero cells by means of the marker protein ERGIC-53 (Schweizer et al., 1990). The ERGIC mediates exocytic protein transport and has a unique protein composition. Upon lowering the culture temperature to 15°C in Vero cells (Schweizer et al., 1990; and this study) or to 16°C in human M cells (Lippincott-Schwartz et al., 1990) ERGIC-53 is concentrated in close vicinity to the Golgi apparatus but does not enter the stacked Golgi (Schweizer, A., Klumperman, J. and Hauri, H.-P., unpublished data). Does this concentration reflect temperature-dependent changes of the ERGIC or a temperature-induced movement of p53 within a stable ERGIC? The results of the present study with p63 argue in favor of a stable ERGIC. This notion is based on the following observations. (I) p63 is specifically localized in smooth membranes, which in the living cell probably form an extended network in the cytoplasm. (II) p63 immunoreactivity was largely absent from rough ER and stacked Golgi apparatus. (III) Contrary to ERGIC-53, p63 is little affected by a 15°C treatment. (IV) All levels of resolution, i.e. conventional immunofluorescence microscopy in conjunction with microtubule perturbation, confocal laser scanning immunofluorescence, as well as immunoelectron microscopy, revealed overlap of the distribution of p63 and ERGIC-53. (V) Newly synthesized VSV G protein has access to the p63 compartment after release from the ER by lowering the temperature from 39.5°C to 15°C. Interestingly, when mAbs against the isolated ERGIC fraction of Vero cells were produced a majority of mAbs exhibited an immunofluorescence pattern

that was similar to the G1/296 pattern and was insensitive to treatment at 15°C (Schweizer, A. Bucher, K. and Hauri, H.-P. unpublished data).

How is the ER-to-Golgi pathway organized?

The present models on the organization of the early secretory pathway from ER to Golgi in higher eukaryotes are contradictory and the structure-function relationship is unclear (Hauri and Schweizer, 1992). The problem arises, on the one hand, from the uncertainty as to whether or not the intermediate compartment is physically separated from rough ER and *cis*-Golgi. In case of a complete separation,

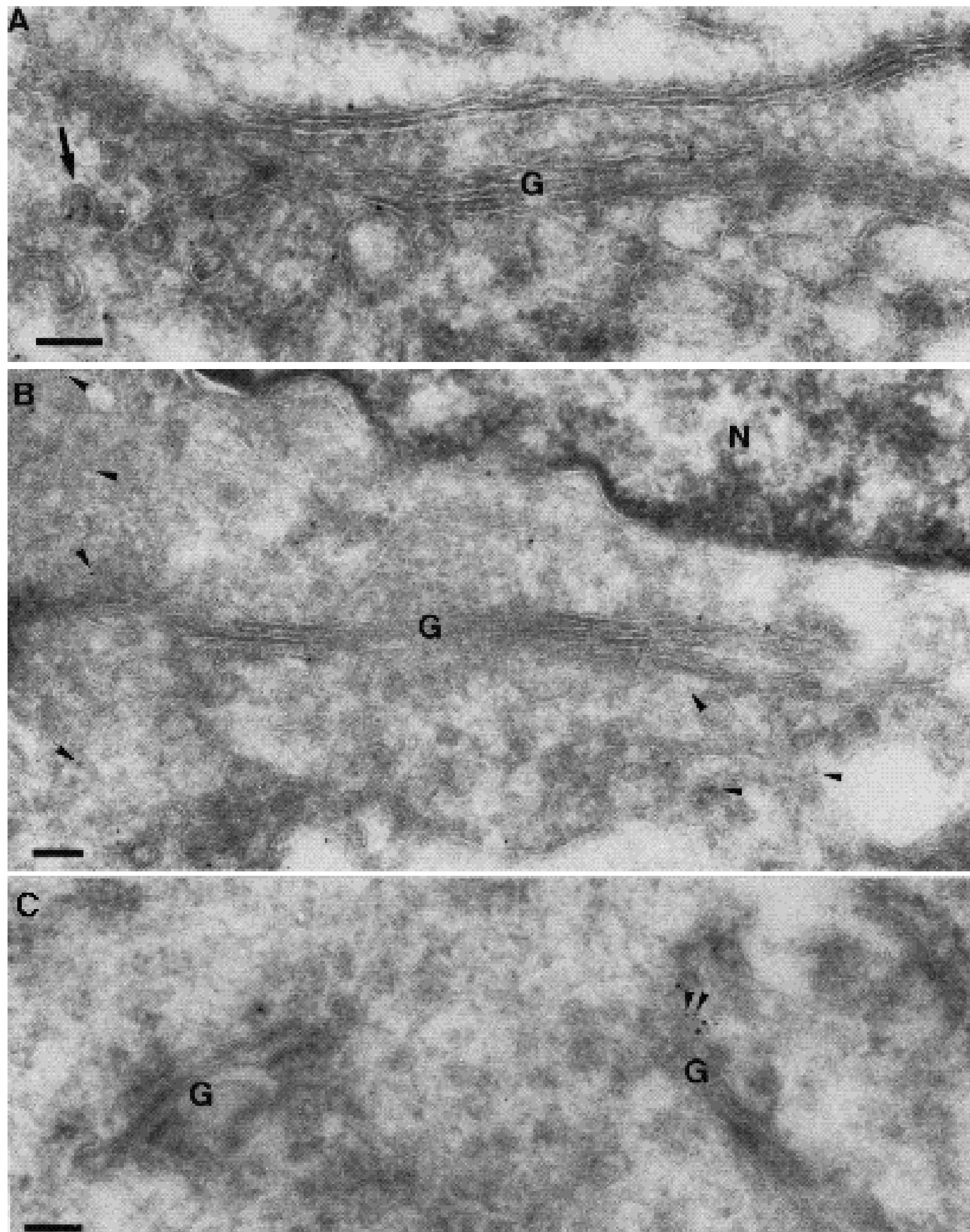

Fig. 7. Ultrastructural localization of p63 in ultrathin cryosections. (A) Single labeling of Vero cells with G1/296 against p63. The labeling is predominantly on one side of the Golgi stack (G). The arrow indicates a coated bud that is labeled. (B and C) Examples of double-labeling with G1/296 against p63 (large gold, unlabeled) and G1/93 against ERGIC-53 (small gold, arrowheads) in the vicinity of the Golgi apparatus. Both markers are predominantly associated with tubulo-vesicular structures close to the Golgi stack (G). In C labeling for both antigens is shown over the same structure in close vicinity to the Golgi stack. N, nucleus. Bars, 100 nm.

two vesicular steps would be required from the rough ER to the *cis*-Golgi. Another problem concerns the identification of the salvage compartment referring to the post-ER site from where retrieval of KDEL-bearing ER proteins occurs (Warren, 1987). In higher eukaryotes the salvage event was initially ascribed to an ER-Golgi intermediate

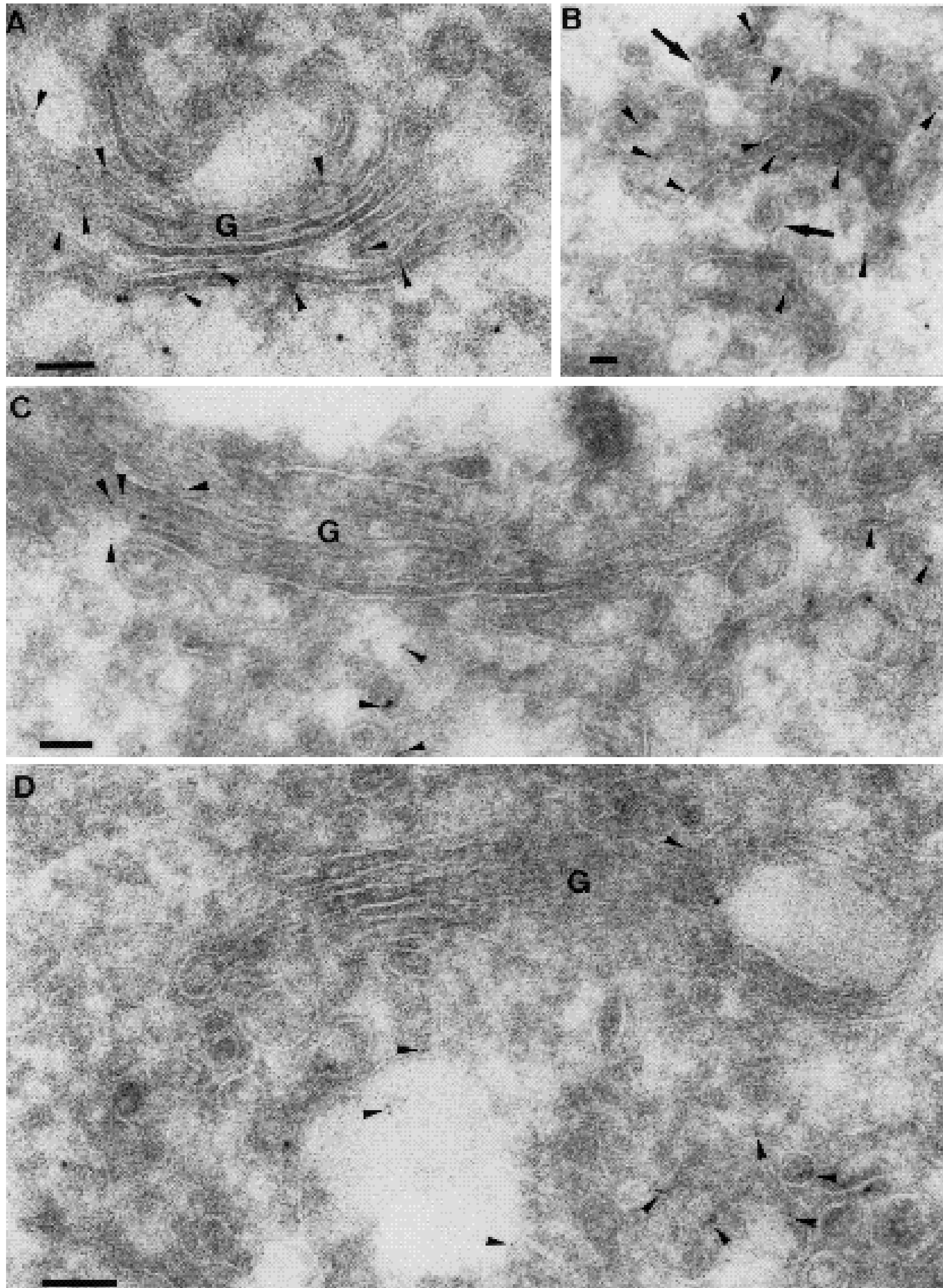

Fig. 8. Co-localization of p63 with the G protein of VSV in ultrathin cryosection of Vero cells blocked for 2 h at 15°C. (A,D,C) Cross-sections through the Golgi apparatus; (B) a section through a tubulo-vesicular cluster. The small gold (arrowheads) labels the G protein and the large gold (unlabeled) labels the p63. Most of the label for both antigens is associated with tubulo-vesicular structures close to the Golgi stack (G). The arrows in B point to coated bud structures, devoid of labeling in continuity with tubulo-vesicular structures that label significantly for the G protein and have one gold particle for p63. Bars, 100 nm.

compartment (Warren, 1987; Pelham, 1989), based on several observations including those from experiments in which the KDEL sequence was attached to the lysosomal enzyme cathepsin D (Pelham, 1988). This construct was modified by the enzyme phosphotransferase, which is believed to function, at least in part, at a post-ER pre-Golgi site, based on subcellular fractionation data (Lazzarino and Gabel, 1988). Subsequent experiments in yeast suggested recycling of HDEL-bearing proteins from an early Golgi compartment (Dean and Pelham, 1990). Accordingly, the human homolog of the yeast 26 kDa HDEL receptor was localized by immunofluorescence to the Golgi apparatus in COS cells (Lewis and Pelham, 1990, 1992). KDEL proteins may, however, cycle as far as from the *trans*-Golgi, as exemplified by CaBP3, an ER resident protein of rat liver, whose N-linked oligosaccharide side chains carry terminal galactose (Peter et al., 1992).

Duden et al. (1991a) and Mellman and Simons (1992) propose that the *cis* osmophilic tubular network of the Golgi apparatus (Lindsey and Ellisman, 1985b; Rambourg and Clermont, 1990), which they refer to as "*cis*-Golgi network", may include the ERGIC-53 compartment and the salvage compartment (Warren, 1987). These authors place the 15°C block between the *cis*-Golgi network and the next *cis*-Golgi cisterna. The term *cis*-Golgi network was also proposed by Hsu et al. (1991) and suggested to operate in the recycling of unassembled MHC class I molecules to the ER. According to this study the *cis*-Golgi network would comprise up to 3 Golgi cisternae.

The most straightforward interpretation of the present study on p63 is as follows. p63 delineates a large tubular network biosynthetically, interposed between rough ER and *cis*-Golgi, that includes the ERGIC defined by ERGIC-53. Based on its general topology and its close apposition to the nuclear membrane, this compartment may be in continuity with the rough ER. However, the labeling intensity for p63 at the ultrastructural level was not strong enough to identify unequivocally rough- to smooth-membrane transitions as continuites between rough ER and the p63 membranes. ERGIC-53 is assumed to be mobile within the p63 network. At 37°C, ERGIC-53 displays a gradient with highest concentrations in the p63 elements close to the Golgi apparatus. This gradient was apparently dissipated in cells incubated at 10°C, resulting in an ERGIC-53 pattern that was very similar to that of p63 (not shown). At 15°C, however, ERGIC-53 concentrated in tubulo-vesicular profiles close to the Golgi apparatus (Schweizer et al., 1990, and this paper). This budding area is illustrated in Fig. 8B. The budding structures carry a nonclathrin coat that labels with an antibody to beta-COP (Griffiths, G., Duden, R. and Kreis, T., unpublished data; Duden et al., 1991a,b). These structures would represent exit sites of the ERGIC where vesicles depart to the Golgi apparatus. Lowering the temperature to 15°C slows down vesicle formation or vesicle fusion with the Golgi apparatus. The occasional presence of ERGIC-53 in the *cis*-Golgi (Schweizer et al., 1988; Chavrier et al., 1990) would reflect a recycling pathway for ERGIC-53 (Lippincott-Schwartz et al., 1990).

In this working model newly synthesized exocytic membrane proteins leave the rough ER at multiple sites by moving within the plane of the membrane into the p63 network. Subsequently the proteins are transported to the Golgi apparatus by a vesicular step. In that sense the ERGIC defined by p63 may be viewed as transitional elements of the rough ER originally described by Palade (1975) for acinus cells of the pancreas with the notable extension that these elements form a permanent network rather than a punctual exit site for export of proteins. Protein movement into and within the ERGIC may require energy as reflected by an early ATP requirement in reconstituting ER-to-Golgi protein transport in semi-intact cells (Beckers et al., 1990). Lowering the temperature to 10°C (Tartakoff, 1986) would inhibit protein transport to the exit site of the ERGIC by a mechanism that is not well understood. Beckers et al. (1990) have also reported an early cytosol requirement before the proteins can be arrested by 15°C. Most likely these factors are needed in priming events for the budding of transport vesicles. The increased number of budding structures observed in the intermediate compartment at 15°C may be a reflection of these priming events. Protein transport from the 15°C compartment is known to require GTP, NSF, ATP, calcium and ARF (Beckers et al., 1989, 1990; Beckers and Balch, 1989; Balch et al., 1992). These factors can be envisioned to be essential for targeting, uncoating and fusion of the ERGIC-to-*cis*-Golgi transport vesicles.

In an alternative model for the organization of the ER-Golgi pathway, which cannot be excluded by our data, p63 would reside in the classical smooth ER (Palade, 1975). The ERGIC, i.e. the membranes with highest levels of ERGIC-53 would not contain p63. Overlap of p63 and ERGIC-53 would result from ERGIC-53 molecules that have recycled to the smooth ER via the Golgi apparatus. This view does not necessarily imply that the ERGIC is an only transiently existing membrane structure as has been proposed by Saraste and Svensson (1991). The partial codistribution of p63 and VSV-G would indicate that VSV G protein has access to smooth ER. In a previous study, Bergmann and Fusco (1990) provided immunofluorescence evidence for such a possibility. These authors even proposed that VSV G protein can egress from the smooth ER of a mutant Chinese hamster cell line UT-1. Exit of VSV G protein from smooth ER could conceivably occur by backflow to the rough ER, followed by entry into the ERGIC, by direct transport from smooth ER to Golgi, or by dynamic interaction between smooth ER and ERGIC, bypassing the rough ER.

Further experiments are required to differentiate between the two models. Such studies should include the preparation of new antibodies against p63 with high labeling efficiency at the ultrastructural level to allow for quantification and double labeling with smooth ER markers. More extended morphological studies will also be necessary to elucidate the nature of the direct continuities observed between the rough ER and the Golgi apparatus (Lindsey and Ellisman, 1985a,b; Tanaka and Fukudome, 1991). Perhaps such continuities are dynamic and represent the recycling pathway from *cis*-Golgi back to the ERGIC (Lippincott-Schwartz et al., 1990; Orci et al., 1991; Schweizer, A., Klumperman, J. and Hauri, H.-P., unpublished data). Furthermore, affinity methods will have to be developed for

the isolation of the different smooth membranes that are interposed in between ER and Golgi.

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