

The Isolated ER-Golgi Intermediate Compartment Exhibits Properties That Are Different from ER and *cis*-Golgi

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Abstract. A procedure has been established in Vero cells for the isolation of an intermediate compartment involved in protein transport from the ER to the Golgi apparatus. The two-step subcellular fractionation procedure consists of Percoll followed by Metrizamide gradient centrifugation. Using the previously characterized p53 as a marker protein, the average enrichment factor of the intermediate compartment was 41. The purified fraction displayed a unique polypeptide pattern. It was largely separated from the rough ER proteins ribophorin I, ribophorin II, BIP, and protein disulfide isomerase, as well as from the putative

cis-Golgi marker *N*-acetylglucosamine-1-phosphodiester- α -*N*-acetylglucosaminidase, the second of the two enzymes generating the lysosomal targeting signal mannose-6-phosphate. The first enzyme, *N*-acetylglucosaminylphosphotransferase, for which previous biochemical evidence had suggested both a pre- and a *cis*-Golgi localization in other cell types, cofractionated with the *cis*-Golgi rather than the intermediate compartment in Vero cells. The results suggest that the intermediate compartment defined by p53 has unique properties and does not exhibit typical features of rough ER and *cis*-Golgi.

NEWLY synthesized exocytic proteins leave the ER at the transitional elements of the rough ER (Palade, 1975) and migrate to the Golgi apparatus through a recently identified intermediate compartment (Saraste and Kuismanen, 1984; Schweizer et al., 1990). This transport intermediate is defined by p53, a 53-kD nonglycosylated homooligomeric transmembrane protein (Schweizer et al., 1988). In Vero cells cultured at 37°C, p53 labels a tubulovesicular membrane system in the vicinity of the *cis*-side of the Golgi apparatus. Newly synthesized G protein was shown to accumulate in the p53 compartment at 15°C demonstrating that p53 identifies an ER-to-Golgi intermediate of the exocytic protein transport pathway of Vero cells (Schweizer et al., 1990).

Besides mediating exocytic protein transport the functions of the ER-Golgi intermediate compartment are unknown. A number of cellular mechanisms have been postulated to occur at a post-ER pre-Golgi site such as salvage of ER components (Warren, 1987; Wieland et al., 1987; Pelham, 1988, 1989; Lippincott-Schwartz et al., 1990; Vaux et al., 1990), the first step of *O*-glycosylation (Tooze et al., 1988), fatty acylation of proteins (Rizzolo et al., 1985; Rizzolo and Kornfeld, 1988; Bonatti et al., 1989) or the first enzymatic step in the generation of the mannose-6-phosphate signal for lysosomal protein targeting (Pelham, 1988; Kornfeld and Mellman, 1989) but precise localization of these functions has been elusive.

One approach to elucidate the characteristics and func-

tions of the p53 compartment is its isolation. Using p53 as a marker protein we have now established a subcellular fractionation procedure combining self-forming Percoll and Metrizamide density gradient centrifugation for the isolation of the ER-Golgi intermediate compartment from Vero cells. The isolated intermediate compartment is largely separable from rough ER and *cis*-Golgi elements suggesting that it has unique properties.

Materials and Methods

Materials

Reagents and supplies were obtained from the following sources. DMEM (4.5 g/liter of glucose), penicillin, streptomycin, fungizone, nonessential amino acids were from Gibco Laboratories (Paisley, Scotland), FCS from Biological Industries (Israel), cell culture dishes from Falcon Labware (Becton Dickinson & Co., Cockeysville, MD), SDS-PAGE reagents from Serva Biochemicals (Heidelberg, FRG), Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden), Metrizamide from Nycomed (Oslo, Norway), trypsin inhibitor and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide from Sigma Chemical Co. (St. Louis, MO), [¹⁴C]uridine-5'-diphospho-D-galactose from New England Nuclear (Boston MA), 4-nitrophenylphosphate disodium salt from Merck (Darmstadt, Germany), nitrocellulose from Schleicher & Schuell Inc. (Keene, NH), powdered milk (Sanolait, Basel) from Coop (Switzerland), fish gelatine from Inotech A. G. (Wohlen, Switzerland), rabbit anti-mouse Ig G1 antibody from Nordic Immunology (Tilburg, The Netherlands), and glutaraldehyde from Fluka Chemical (Buchs, Switzerland). Protein A was iodinated with Na¹²⁵I by the chloramine T procedure.

Cell Culture

Vero cells (African green monkey kidney cells) were grown in Optilux petri

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dishes (Becton Dickinson Co., Lincoln Park, NJ) in DMEM supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml fungizone under standard tissue culture conditions.

Antibodies

Mouse mAb G1/93 against the 53-kD protein has previously been characterized (Schweizer et al., 1988). A polyclonal antibody against the denatured 53-kD protein was produced as follows. The 53-kD protein was immunoprecipitated from Caco-2 membranes using mAb G1/93 and the immunoprecipitate was subjected to SDS-PAGE. The p53 band was cut out of the gel, homogenized in PBS with a glass-Teflon potter, and mixed with complete Freund's adjuvant. The resulting emulsion was subcutaneously injected into a New Zealand White rabbit. Booster injections with antigen in incomplete Freund's adjuvant were given 1, 5, and 12 mo later by the same route. 1 wk after the third booster the rabbit was killed and the serum was harvested. Mouse mAbs directed against ribophorin I and II (Hortsch et al., 1986) were a kind gift of Dr. D. Meyer (University of California, Los Angeles). Rabbit polyclonal antibodies to protein disulfide isomerase and BIP (Vaux et al., 1990) were generously provided by Dr. S. Fuller (European Molecular Biology Laboratory); they were raised against peptides containing the carboxy-terminal tail of either protein disulfide isomerase (KDDDKAVKDEL) or BIP (KEEDTSEKDEL).

Isolation of the p53 Compartment (Fig. 1)

All the steps of the isolation procedure were carried out at 4°C unless indicated otherwise. Vero cells (12 100-mm petri dishes per preparation) were washed once with 0.9% (wt/vol) NaCl and once with 250 mM sucrose, 10 mM triethanolamine-acetic acid, pH 7.4 (buffer A). The cells were then scraped from the dishes, suspended in 2 ml per three 100-mm dishes 250 mM sucrose, 1 mM Na₂EDTA, 10 mM triethanolamine-acetic acid, pH 7.4 (buffer B), and centrifuged for 5 min at 560 *g*_{av}. The cell pellet was gently resuspended in 2 ml of buffer B and centrifuged as above. The cells were then resuspended in 3 ml of buffer B and homogenized by passing them 10 times through a ball-bearing homogenizer (Balch and Rothman, 1985) with a clearance of 20 µm. The resulting homogenates were pooled and centrifuged for 10 min at 2,000 rpm (370 *g*_{av}) in an SS34 rotor (Sorvall Instruments Division, Norwalk, CT). The supernatant was brought to exactly 30 ml with buffer B and 3.96 ml stock isoosmotic Percoll (density of Percoll = 1.129, initial density = 1.046) were added. The Percoll gradient was centrifuged for 41 min at 20,000 rpm (36,900 *g*_{av}) in a RC2B centrifuge (Sorvall Instruments Division) using the same rotor as above. For the determination of marker enzyme activities and p53 distribution of this Percoll gradient, 23 fractions were collected beginning at the bottom. For the isolation of the p53 compartment 7.5 ml from the bottom of the gradient were discarded. The next 13.5 ml were pooled and processed for two identical gradients as follows: 6.75 g of this fraction were adjusted to 30% (wt/wt) Metrizamide and overlaid with 3.5 ml of each 27% and 18.5% (wt/wt) Metrizamide in 1 mM Na₂EDTA, 10 mM triethanolamine-acetic acid, pH 6.5 (buffer C). Finally the tube was filled with buffer B. The gradient was run to equilibrium (18–20 h) at 25,000 rpm (85,800 *g*_{av}) in a TST 28.17 rotor (Kontron Electronic GmbH, Zurich, Switzerland) at 8°C. The 27%:18.5% interphase was harvested, diluted with PBS and centrifuged at 39,000 rpm (105,000 *g*_{av}) for 1 h at 4°C in a TFT 65.13 rotor (Kontron Electronic GmbH). The pellet was resuspended in PBS. This fraction was designated F3.

Enzyme Assays

The following marker enzymes were used: alkaline phosphatase (measured according to Stieger et al., 1986) for the plasma membrane, glucosaminidase (measured according to Scalera et al., 1980) for lysosomes, galactosyltransferase (measured according to Venable and Coggeshall, 1965; Stieger et al., 1988) for *trans*-Golgi and KCN-resistant NADH oxidoreductase (measured according to Sottocasa et al., 1967) for the ER. Protein was determined with the Bio-Rad protein assay kit using protein standard I (Bio-Rad Laboratories Munchen, Germany).

UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (*N*-acetylglucosaminylphosphotransferase, phosphotransferase) was measured as follows: [β -³²P]uridine diphospho-*N*-acetylglucosamine ([β -³²P]UDPGlcNAc) was synthesized according to the method of Lang and Kornfeld (1984). *N*-acetylglucosaminylphosphotransferase

1. *Abbreviations used in this paper:* BIP, immunoglobulin-binding protein; GlcNAc, *N*-acetylglucosamine.

assays were carried out as previously described (Reitman et al., 1984). [β -³²P]UDP GlcNAc was used at 50–100 µM with a specific activity of 133–400 cpm/pmol and methyl- α -D-mannopyranoside at 100 mM was used as acceptor. One unit of activity is defined as 1 pmol GlcNAc-phosphate transferred per hour.

The assay for *N*-acetylglucosamine-1-phosphodiester- α -*N*-acetylglucosaminidase (uncovering enzyme) was as follows: The substrate [6 -³H]*N*-acetylglucosamine 1'-(α -D-methylmannopyranose 6-monophosphate), was prepared to a specific activity of 1,500 cpm/pmol essentially as described previously (Tong et al., 1989). Enzyme samples and [6 -³H]*N*-acetylglucosamine 1'-(α -D-methylmannopyranose 6-monophosphate) (at 0.12–0.24 mM) were incubated in 50 mM Tris-maleate, pH 6.8, 0.5% (wt/vol) Triton X-100 and 5 mg/ml BSA at 37°C for 2 h. The 25–50-µl reaction was stopped by the addition of 1 ml 5 mM EDTA, pH 7.8. Samples were then loaded onto 0.5 × 1.5 cm quaternary aminoethyl Sephadex columns that had been equilibrated in 2 mM Tris base. Liberated [6 -³H]GlcNAc was collected by washing with 2 mM Tris base and detected by liquid scintillation counting. One unit of activity is defined as 1 pmol GlcNAc released per hour.

Quantification of p53 in Subcellular Fractions

The quantification of p53 was based on a dot-blot procedure (Hawkes et al., 1982) and performed as follows: from the cell fractions to be assayed 4, 2, 1, and 1 µl of twofold serial dilutions in PBS were dotted onto a nitrocellulose filter. After air drying overnight and blocking with 1% powdered milk in PBS, the dots were sequentially incubated for 2 h each with an IgG fraction of mAb G1/93 (5 µg per dot in blocking solution), a rabbit anti-mouse Ig G1 antibody (1:500 in blocking solution and ¹²⁵I-protein A (500,000 cpm per dot). Labeled dots were visualized by autoradiography using Fuji RX films. Quantification of autoradiograms was carried out by means of a Molecular Dynamics 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA). The data of the radiometric assay were analyzed by computer as described by Gorr et al. (1988).

SDS-PAGE and Immunoblotting

Proteins were separated on 8.5% SDS-polyacrylamide slab gels using the Laemmli system (Laemmli, 1970) and stained with Coomassie brilliant blue or transferred to nitrocellulose sheets according to the method of Towbin et al. (1979). For the immunoreaction the nitrocellulose sheet was incubated with the antibody of choice followed by ¹²⁵I-protein A in the presence of PBS-3% fish gelatine-0.05% NP-40 for the anti p53 polyclonal antibody or in the presence of PBS-1% powdered milk for all other antibodies (Hauri and Bucher, 1986).

Electron Microscopy

A sample of the F3 fraction was prefixed at room temperature by adding

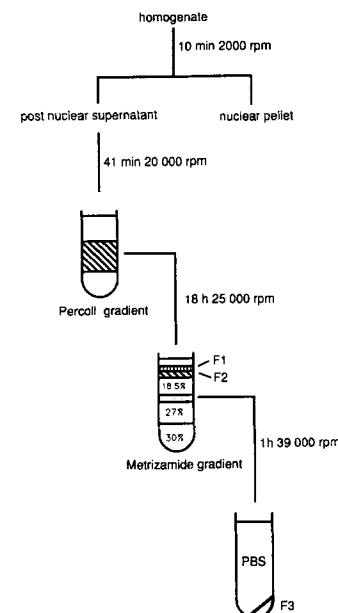


Figure 1. Flow diagram for the isolation of the p53 compartment fraction (F3).

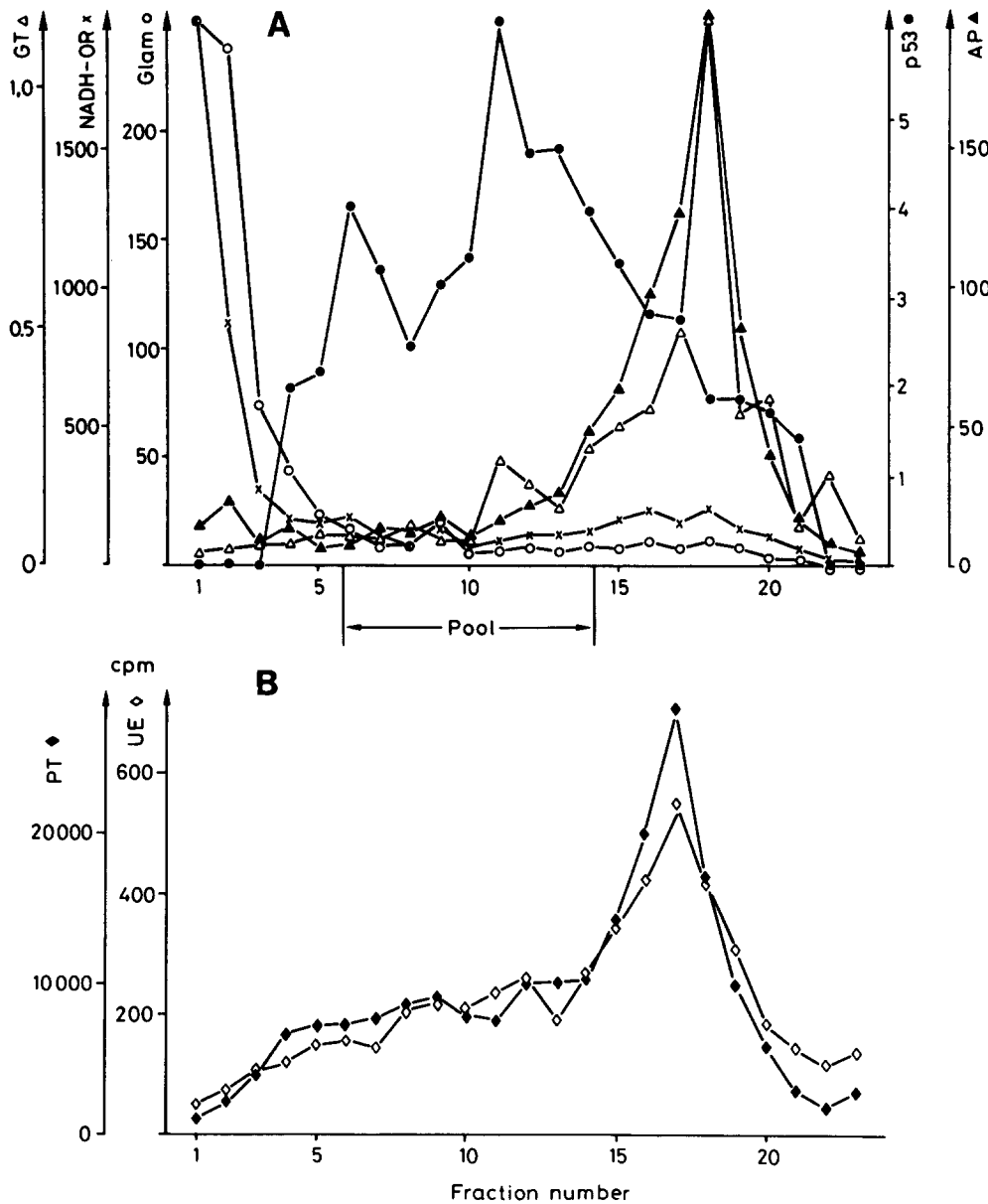


Figure 2. Distribution of p53 and marker enzymes on Percoll gradients. Postnuclear supernatants from Vero cells were fractionated on 10.5% Percoll gradients. The gradient was divided into 23 fractions of 1.5 ml beginning at the bottom. (A) Enzymatic activities of alkaline phosphatase (AP), glucosaminidase (Glam), galactosyltransferase (GT), and KCN-resistant NADH oxidoreductase (NADH-OR) are given in milliunits/milliliter. P53 was detected by immunoblotting; values are given in arbitrary units/milliliter. (B) Enzymatic activities of *N*-acetylglucosaminylphosphotransferase (PT) and uncovering enzyme (UE) are given in counts per minute.

a stock solution of glutaraldehyde (8%) to a final concentration of 1%. After 15 min the samples were stored at 4°C until further processing. The fractions were then suspended for 3 h at 4°C in PBS containing 1% glutaraldehyde. The fixed material was centrifuged in an airfuge (Beckman Instruments, Fullerton, CA) for 15 min at 150,000 g_{av} . Pooled pellets were postfixed for 30 min at 4°C in a 2% solution of osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). The postfixed material was dehydrated and embedded in Epon/araldite according to standard procedures. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 400 transmission electron microscope.

Results

Two-Step Isolation Procedure of the p53 Intermediate Compartment

Vero cells were used as a starting material for the purification of the p53 compartment. This cell line was chosen based on our previous study (Schweizer et al., 1990) which established the p53 compartment as an intermediate of the ER-to-Golgi

protein transport pathway. Since mAb G1/93 recognizes an epitope on the extracytoplasmic side of p53 (Schweizer et al., 1988) it was not possible to isolate the p53 compartment by affinity methods. Therefore the isolation was based on self-forming Percoll and Metrizamide equilibrium density gradients using p53 as a marker protein (Fig. 1).

The first step of the isolation procedure was derived from pilot studies showing that the p53 compartment displayed an intermediate density on self-forming Percoll gradients when compared with ER and Golgi markers. The Percoll gradient of a postnuclear supernatant from Vero homogenates was optimized in such a way that the dense organelles such as ER (defined by KCN-resistant NADH-oxidoreductase activity) and lysosomes (defined by glucosaminidase activity) ran close to the bottom of the gradient, whereas the less dense organelles, i.e., *trans*-Golgi (defined by galactosyltransferase activity) and plasma membrane (defined by alkaline phosphatase activity) ran close to the top of the gradient. The separation was optimal with 10.5% Percoll (Fig. 2 a).

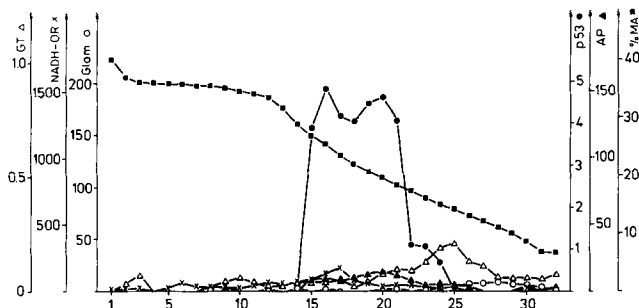


Figure 3. Distribution of p53 and marker enzymes on a continuous Metrizamide gradient. Postnuclear supernatants of Vero cells were fractionated on a 10.5% Percoll gradient as shown in Fig. 2. Fractions 6–14 (pool in Fig. 2) were further fractionated on a 5–30% Metrizamide gradient. The gradient was divided into 32 fractions of 1.05-ml beginning at the bottom. Symbols and activities are as in Fig. 2; filled squares indicate the concentration of Metrizamide (wt/wt).

On such gradients p53 was found in the center as a broad peak when analyzed by immunoblotting of gradient fractions with a polyclonal antibody raised against the denatured p53.

Fractions 6–14 were pooled and served as starting material for the second purification step. For this purpose the pooled fractions were subjected to equilibrium flotation on a discontinuous Metrizamide gradient that was optimized according to the sedimentation properties of the p53 compartment on continuous Metrizamide gradients (Fig. 3). The 18.5%/27% interphase was collected and subjected to a high-speed centrifugation in PBS. This final fraction was designated F3. The marker characterization of this fraction is given in Tables I and II. To measure quantitatively p53 in subcellular fractions of Vero cells a dot-blot procedure using mAb G1/93 was developed (see Materials and Methods for detail). Based on this determination F3 was found to be enriched 40.9-fold in p53 with a yield of 29%. None of the other organelle markers were enriched with the exception of the ER marker KCN-resistant NADH-oxidoreductase which was twofold enriched. The recovery for all the markers was between 69 and 108%. Based on this biochemical analysis we conclude that the procedure depicted in Fig. 1 efficiently separates the p53 compartment from *trans*-Golgi, plasma membrane, lysosomes, and ER.

Table I. Specific Activities and Enrichment Factors of p53 and Marker Enzymes in F3

	Specific activity*		F3 enrichment factor
	Homogenate	F3	
53-kD protein	0.9 ± 0.5	33.2 ± 13.1	40.9 ± 12.2
Glucosaminidase	21.1 ± 4.6	6.8 ± 3.2	0.3 ± 0.2
Galactosyl-transferase	0.11 ± 0.02	0.07 ± 0.03	0.6 ± 0.2
KCN-resistant NADH oxidoreductase	331 ± 72	688 ± 293	2.1 ± 0.8
Alkaline phosphatase	11.7 ± 2.1	5.2 ± 0.9	0.4 ± 0.1

The numbers indicate means ± 1 SD of six independent experiments.

* The values are given in arbitrary units/milligram protein for p53 and milliunits/milligram protein for the enzymes.

Table II. Yield and Recovery of Marker Enzymes in a Fraction Enriched in p53 (F3)

	Total*	F3
Protein	87 ± 11	0.8 ± 0.2
53-kD protein	84 ± 8	28.6 ± 7.7
Glucosaminidase	99 ± 12	0.3 ± 0.1
Galactosyltransferase	108 ± 13	0.5 ± 0.2
KCN-resistant NADH oxidoreductase	69 ± 2	1.9 ± 0.7
Alkaline phosphatase	82 ± 7	0.3 ± 0.1

The values are given as percentage of the amount determined in the homogenate and represent means ± 1 SD of six independent experiments.

* Sum of the amount recovered in each fraction.

Morphological Characterization of the Isolated p53 Compartment

Transmission EM was performed to characterize morphologically the F3 fraction (Fig. 4). The fraction was found to be almost exclusively composed of numerous smooth-surfaced membrane vesicles. Many vesicles with an average diameter of 89 ± 18 nm (mean ± 1 SD, $n = 95$) exhibited an electron-dense core, whereas some larger vesicles were empty. The dense core structures which probably represent vesicles carrying secretory products may correspond to the dense-core vesicles that stain positive with the anti-p53 antibody in ultrathin cryosections of various cells (Schweizer et al., 1988, 1990), whereas the larger vesicles may originate from the tubular elements of the p53 compartment. The vesicles had no obvious electron-dense coat but many of them showed a dark staining-limiting membrane. It is worth noting that the F3 fraction was free from drumstick-like profiles characteristic for Golgi cisternae (Stieger et al., 1988) and from endosomes (Gruenberg et al., 1989; McDowall et al., 1989) which can be expected to remain intact, at least in part, considering the rather mild homogenization conditions used. These conditions were identical to those used for the isolation of the Golgi apparatus in Stieger et al. (1988). Furthermore, our previous experiments have shown that endosomes as defined by a 15-min uptake of horseradish peroxidase display low density on Percoll gradients, a density that was even slightly lower than that of the plasma membrane (Matter et al., 1990).

Polypeptide Composition of the Isolated p53 Compartment

Comparative SDS-PAGE of subcellular fractions (Fig. 5) revealed the enrichment of at least 16 bands when compared with the homogenate (compare lanes 1 and 6). These proteins display molecular masses between 23 and 149 kD. The position of p53 was identified by immunoblotting (not shown). This protein pattern is similarly complex as that of purified Golgi apparatus (Malhotra et al., 1989) but less complex than that of rough endoplasmic reticulum (Amar-Costesec et al., 1988). These data suggest that the F3 fraction contains a distinct set of proteins.

Relationship of the Isolated p53 Compartment to Rough ER and *cis*-Golgi

Among the best characterized proteins known to be restricted to the rough ER are the integral membrane proteins

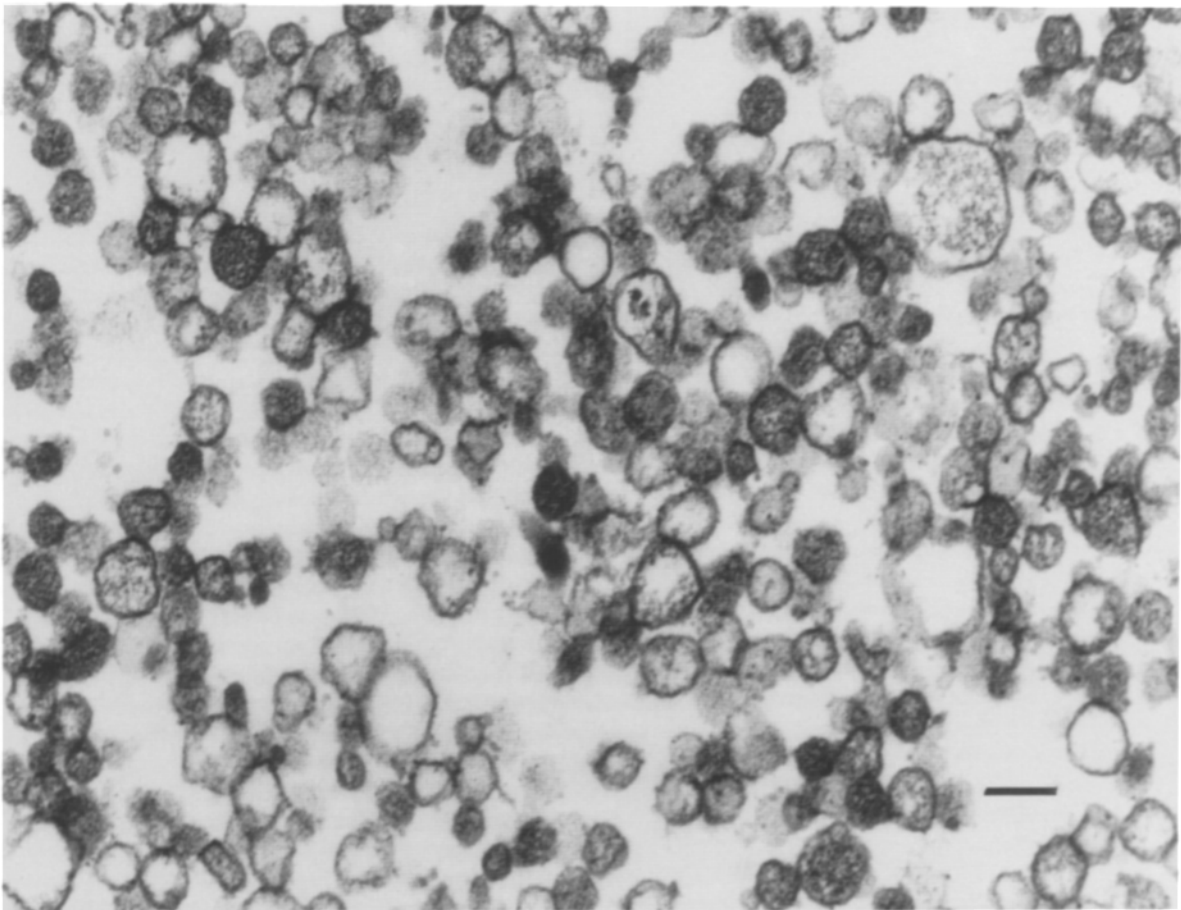
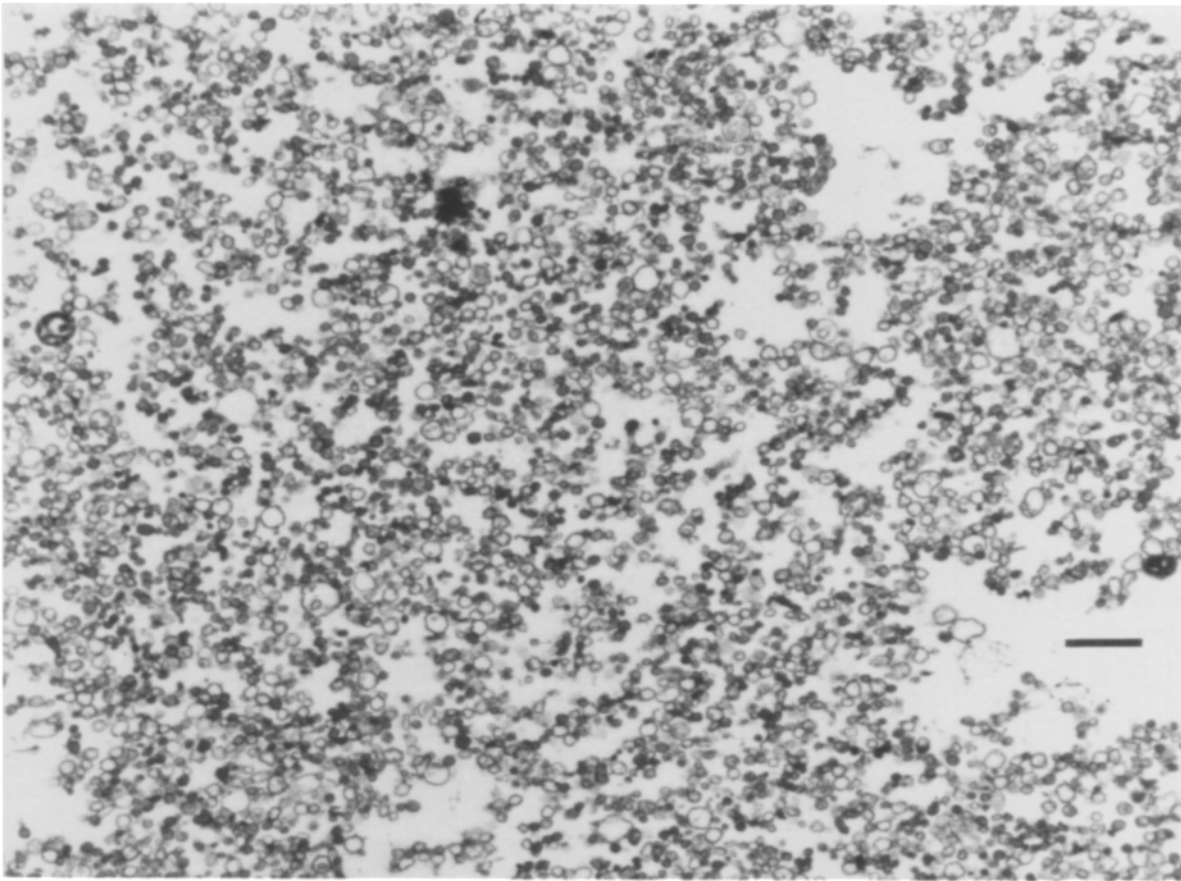


Figure 4. Representative transmission electron micrograph of the p53 compartment fraction F3. Bars: (top) 0.83 μm ; (bottom) 0.18 μm .

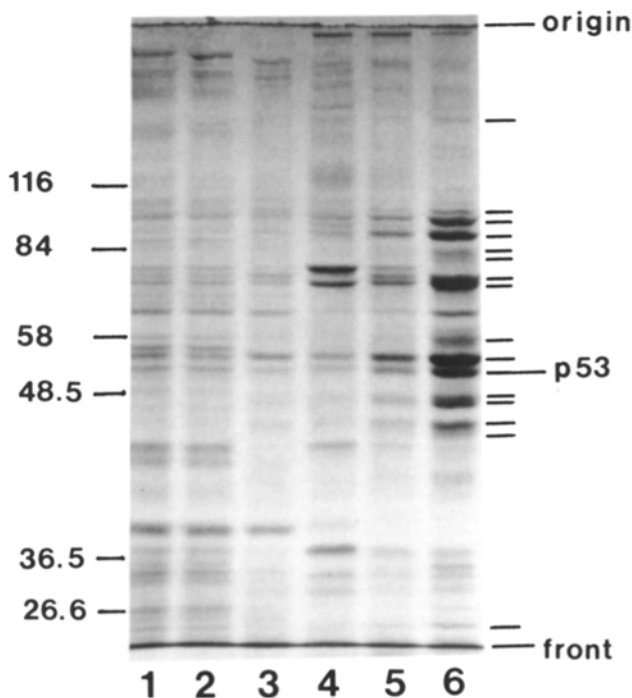


Figure 5. Comparative polypeptide composition of subcellular fractions. Aliquots of subcellular fractions (100 μ g protein/lane) were subjected to SDS-PAGE (8.5% gel) and stained with Coomassie brilliant blue. Lane 1, homogenate; lane 2, postnuclear supernatant; lane 3, pooled fractions 6–14 of Percoll gradient; lane 4, F1 (see Fig. 1), lane 5, F2 (see Fig. 1), and lane 6, F3. Molecular weights were determined from a set of markers (not shown). The bars at the right margin indicate protein bands specifically enriched in the F3 fraction.

ribophorin I (65 kD) and II (two protein subunits, \sim 63 kD) (Kreibich et al., 1978; Hortsch et al., 1986; Crimando et al., 1987), which were initially proposed to mediate the attachment of ribosomes but whose function is still unclear. Soluble resident ER proteins include the 57-kD enzyme protein disulfide isomerase, which catalyzes the rearrangement of disulfide bonds (Freedman, 1984; Edman et al., 1985) and the 78-kD protein BIP (glucose-regulated protein 78) (Bole et al., 1986, 1989; Munro and Pelham, 1986), which may function in protein folding and oligomerization. To investigate to what extent the isolated p53 compartment is related to the rough ER, Western blots of Vero homogenates and corresponding F3 fractions having equal protein amounts were probed with mAbs against ribophorin I (Fig. 6, lanes 1 and 2) and II (Fig. 6, lanes 3 and 4) and with polyclonal antibodies to protein disulfide isomerase (Fig. 6, lanes 5 and 6) and BIP (Fig. 6, lanes 7 and 8). Quantification of the immunoblots by densitometric scanning gave the following enrichment factors (mean \pm 1 SD, $n = 3$). Ribophorin I: 1.4 ± 0.4 ; ribophorin II: 1.1 ± 0.1 ; protein disulfide isomerase: 1.8 ± 0.3 ; BIP: 2.8 ± 0.4 . Thus, the markers were, if at all, only slightly enriched comparable to the enrichment of the ER enzyme KCN-resistant NADH oxidoreductase. These results suggest that we have largely separated the p53 compartment from the rough ER.

To date, no accepted *cis*-Golgi marker protein is known

whose localization has been confirmed by immunoelectron microscopy. Candidate *cis*-Golgi markers as deduced from biochemical studies include Golgi α -mannosidase IA (Tabas and Kornfeld, 1979; Dunphy and Rothman, 1983), *N*-acetylglucosaminylphosphotransferase (phosphotransferase) (Goldberg and Kornfeld, 1983; Kornfeld and Kornfeld, 1985) and the uncovering enzyme *N*-acetylglucosamine 1-phosphodiester- α -*N*-acetylglucosaminidase (Goldberg and Kornfeld, 1983; Kornfeld and Kornfeld, 1985; Lazzarino and Gabel, 1988, 1989). The latter two enzymes are responsible for the formation of the mannose-6-phosphate targeting signal of lysosomal enzymes (Kornfeld and Mellman, 1989). Rabbit antibodies against rat liver Golgi mannosidase IA (Tulsiani and Touster, 1988) were unable to recognize the corresponding enzyme in the monkey-derived Vero cells. Measurements of this enzyme activity are complicated by possible interference with other mannosidases (Bishoff and Kornfeld, 1983; Bishoff et al., 1986). We decided therefore to measure the distribution of phosphotransferase and uncovering enzyme activities in the fractions of the Percoll gradient (Fig. 2 *b*) and in the F3 fraction (Table III) to investigate the relationship of the p53 compartment and *cis*-Golgi. Both enzymes comigrated to the same position on the Percoll gradient close to the galactosyltransferase peak. The peak fractions of both phosphotransferase and uncovering enzyme were localized to one fraction denser than the peak of the *trans*-Golgi marker galactosyltransferase. There was also some trailing of the phosphotransferase and uncovering enzyme activities into the broad p53 peak. Table III shows that in the F3 fraction phosphotransferase was enriched five- to sixfold and uncovering enzyme only two- to three fold. This result suggests that the major localization of phosphotransferase and the uncovering enzyme in Vero cells is not the p53 intermediate compartment but most likely the *cis*-Golgi. The p53 compartment therefore can also be separated from the *cis*-Golgi elements.

Discussion

The recently described 53-kD protein has been shown to be a marker for an ER-Golgi intermediate of the exocytic transport pathway in Vero cells (Schweizer et al., 1988, 1990). This p53 compartment has now been purified by a combination of Percoll and Metrizamide gradient centrifugation resulting in an efficient separation from ER, Golgi, lysosomes and plasma membrane markers and a 41-fold enrichment of p53. Despite this considerable enrichment we are aware of the possibility that further purification may be required before the p53 compartment can be considered free of any contaminating membranes for which there are no accepted markers. As soon as sequence information of the cytoplasmic domain of p53 is available antibody tools can be generated for a more complete purification of the intermediate compartment by immunoaffinity procedures. Nevertheless, the achieved degree of purification already allows important conclusions concerning the relationship of the p53 compartment with its neighbour organelles, the ER and the Golgi apparatus as discussed below.

Electron micrographs of the isolated fraction demonstrate the presence of numerous smooth vesicles. Many of the vesicles with an average diameter of 89 nm exhibit an electron dense interior and resemble the vesicular structures stained

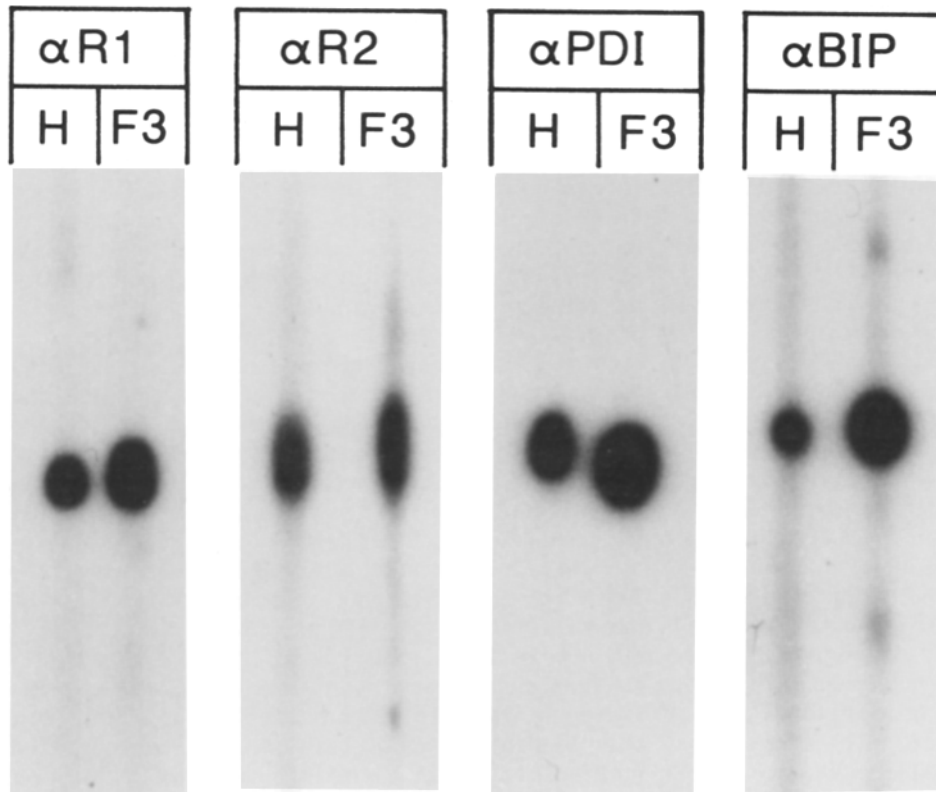


Figure 6. Immunoblot analysis of homogenate (H) and F3 fractions with antibodies to rough ER markers (autoradiograms). Homogenate and F3 were prepared as described and equal protein amounts (22 $\mu\text{g}/\text{mm}$ gel width) were subjected to SDS-PAGE (8.5% gel) and immunoblotting. Individual nitrocellulose strips were probed with a mAb against ribophorin I ($\alpha R1$); a mAb against ribophorin II ($\alpha R2$); a polyclonal antibody against protein disulfide isomerase (αPDI); or a polyclonal antibody against protein BIP (αBIP). Only the area with the immunoreaction is shown. BIP was consistently more enriched in the F3 fraction than ribophorin I, ribophorin II or disulfide isomerase. The apparent shift of the αPDI immunoreaction in F3 versus homogenate is due to nonperfect alignment of the two nitrocellulose strips in the film cassette. Ribophorin II is composed of two subunit proteins (Crimaudo et al., 1987), which were not resolved as two discrete bands in our blotting procedure at the exposition time used.

with the anti-p53 antibody on cryosections in situ. However, the thin (apparently nonclathrin) coat which is present on at least some p53-bearing vesicles in situ (Schweizer et al., 1988) could not unambiguously be detected in the isolated fraction. This may reflect some technical problems in the preparation of the isolated fraction for EM or represent a loss of the coat. Jamieson and Palade (1967) have described smooth surface vesicles on the *cis* side of the Golgi apparatus in situ. In guinea pig pancreatic cells these vesicles were found to have a diameter of about 50 nm and were covered by a fine fibrillar coat. Transition vesicles between ER and Golgi have also been depicted by Morr  and colleagues (Nowack et al., 1987) as electron dense structures with a naplike surface coat and a diameter which varies from 55 to 88 nm according to the conditions used (Morr  et al., 1989). The

purification of these vesicular intermediates from a cell-free transport system has been reported (Paulik et al., 1988). Moreover, Lodish et al. (1987) described the isolation of a low-density membrane fraction that has some of the biochemical characteristics expected for ER to Golgi transport vesicles. Recently a vesicular ER-Golgi transport intermediate has also been isolated from yeast (Groesch et al., 1990). This intermediate contained a post-ER/pre-Golgi form of pro- α -factor. All these preparations, however, lack extensive purification and biochemical characterization rendering a comparison with the present F3 fraction impossible.

Investigation of the polypeptide composition of the isolated p53 compartment revealed several prominent bands which were enriched when compared to the homogenate. We do not know the identity of these proteins yet. Importantly, the protein pattern is significantly different from that described for isolated Golgi membranes (Malhotra et al., 1989) or rough ER (Amar-Costesec et al., 1988) suggesting that the isolated fraction has unique properties not shared by its neighbor organelles. Compared with the p53-compartment the polypeptide composition of the 106 nm non-clathrin-coated intra-Golgi vesicles (Malhotra et al., 1989) as well as of the 84 nm noncoated post-Golgi vesicles (de Curtis and Simons, 1989) is less complex in nature. Assuming that protein transport from ER to Golgi is mediated by transport vesicles only and that no tubular intermediate compartment would exist this comparison would suggest that the fraction we have isolated is still contaminated with unknown membranes. An alternative view, and the one we prefer, is that a tubular intermediate compartment exists. This notion re-

Table III. Specific Activities and Enrichment Factors of Putative *cis*-Golgi Markers in F3

		Specific activity*		F3 enrichment factor
		Homogenate	F3	
Phospho-transferase	Exp. 1	110	596	5.4
	Exp. 2	116	703	6.1
Uncovering enzyme	Exp. 1	863	2,490	2.9
	Exp. 2	1,652	4,000	2.4

* The values are given in milliunits/milligram protein; two independent experiments.

sides on the following: first, mAb G1/93 against p53 labels tubular and vesicular profiles at the ultrastructural levels in situ (Schweizer et al., 1988). Second, if the intracellular protein transport to the Golgi apparatus of VSV G protein is arrested at 15°C, the G protein accumulates in tubular and vesicular profiles close to the Golgi apparatus (Schweizer et al., 1990), suggesting that the tubular elements are part of the exocytic pathway. For a membrane compartment, a polypeptide pattern of the complexity we have obtained is not surprising and may largely reflect the protein complement of the intermediate compartment itself. This notion is supported by recent results showing that the majority of mAbs generated against the isolated F3 fraction exhibit immunofluorescence patterns that resemble those obtained with the G1/93 mAb against p53 (Schweizer, A., unpublished).

The isolated p53 compartment was further characterized with respect to its relationship to the rough ER. The two rough ER membrane markers ribophorin I and II and the KDEL-bearing luminal rough ER markers protein disulfide isomerase and BIP were largely separable from the intermediate p53 compartment. This feature suggests that the intermediate compartment may be considered a separate organelle. This notion is further supported by the specific association of a unique low-molecular weight GTP-binding protein related to rab 2 with the p53 compartment in situ (Chavrier et al., 1990). Whether the intermediate compartment is nevertheless connected with the rough ER by membrane continuities as the smooth ER in hepatocytes or whether the two compartments are connected by a vesicular pathway remains to be shown by detailed morphological studies at the ultrastructural level. Since protein disulfide isomerase and BIP have access to a post-ER compartment and are thought to recycle back to the ER by means of the KDEL mechanism (Pelham, 1988) it is not absolutely clear if the slight enrichment of these two proteins in the purified F3 fraction reflects the specific origin of this compartment or simply minor cross-contamination with ER. What is the relationship of the p53 intermediate compartment and the *cis*-Golgi apparatus? Morphologically, what is normally considered the *cis*-most element of the Golgi apparatus appears as a fenestrated cisterna or network in the transmission electron microscope (Rambourg and Clermont, 1990). However, some authors have described direct membrane continuities between *cis*-Golgi cisternae and rough ER (Lindsey and Ellisman, 1985), which complicates the structural definition of *cis*-Golgi. Likewise, in functional terms, the definition of *cis*-Golgi is not clear either because none of the biochemically established putative *cis*-Golgi enzyme markers have been localized yet by immunoelectron microscopy. In the absence of a firm structural-functional relationship, the definition of *cis*-Golgi is tentative. For the present study, we have considered the uncovering enzyme a marker for the *cis*-Golgi.

The initial phosphorylation of lysosomal enzymes by phosphotransferase has been proposed to occur in a post-ER pre-Golgi site (Pelham, 1988; Lazzarino and Gabel, 1988, 1989), while the conversion of monophosphorylated species to diphosphorylated forms by phosphotransferase and the hydrolysis of diesters by the uncovering enzyme is believed to occur within the *cis*-Golgi (Lazzarino and Gabel, 1988, 1989; Kornfeld and Mellman, 1989). On our Percoll gradients the main peaks of phosphotransferase and uncovering

enzyme were unseparable from one another but they did not colocalize with the p53 peak. In the final F3 fraction the relative enrichment was 5 to 6 for phosphotransferase and 2 to 3 for the uncovering enzyme, considerably less than the enrichment for p53. Given the above series of phosphorylation events one possible explanation for these results is that phosphotransferase has a biphasic distribution with a minor portion in the p53 compartment and the major portion in the *cis*-Golgi. Alternatively, both enzymes may be localized beyond the intermediate compartment in Vero cells and the activity in the F3 fraction may just reflect cross-contamination with elements of the *cis*-Golgi. It is worth noting, however, that the enrichment in F3 was always consistently higher for phosphotransferase than for the uncovering enzyme.

On the Percoll gradient there was some trailing of p53 into the main phosphotransferase/uncovering enzyme peak that may be related to the finding in other human cells that the first fenestrated *cis*-Golgi cisterna showed weak immunoreactivity with the mAb against p53 (Schweizer et al., 1988; Chavrier et al., 1990). p53 may have access to the *cis*-Golgi and there is immunofluorescence evidence for recycling of p53 to the ER in human M1 cells (Lippincott-Schwartz et al., 1990). Therefore, p53 cannot a priori be considered an absolute marker for the ER-Golgi intermediate compartment in all cell types. In Vero cells, however, the majority of p53 resides in the intermediate compartment as assessed by labeling studies with mAb G1/93 (Schweizer et al., 1990). This observation was in fact the basis for using Vero cells in the present study. Collectively, the results suggest to us that we have largely separated the p53 compartment from *cis*-Golgi elements defined by the uncovering enzyme. As with the rough ER this does not necessarily imply absence of membrane continuities. It should be noted, however, that the p53 compartment behaves differently from *cis*-Golgi (defined by the putative *cis*-Golgi marker mannosidase IA and the *cis*-medial Golgi marker mannosidase II) in cells that have been treated with organelle perturbants such as brefeldin A or nocodazole (Lippincott-Schwartz et al., 1989, 1990).

Using semi-intact cells to reconstitute ER to Golgi transport in a cell-free system, Balch and co-workers established a number of biochemical requirements for ER to *cis*-Golgi protein transport (Beckers et al., 1987; Balch, 1989; Beckers and Balch, 1989). In particular they postulate the existence of an intermediate compartment where transport is inhibited by GTP γ S, EGTA, or *N*-ethylmaleimide. It will be of interest to study the functional properties of the isolated p53 compartment in respect to these findings.

Given the successful isolation of the p53 intermediate compartment it should now be possible to study its functions in more detail. Candidate functions include all processes which have been assigned to a post-ER pre-Golgi site, such as the addition of *N*-acetylgalactosamine to serine and threonine residues of glycoproteins (Tooze et al., 1988), fatty acylation of proteins (Rizzolo et al., 1985; Rizzolo and Kornfeld, 1988; Bonatti et al., 1989) and binding of endogenous antigens to class I histocompatibility antigens (Nuchtern et al., 1989; Yewdell and Bennik, 1989; Cox et al., 1990). An additional possible function of the p53 compartment might be the salvage of luminal ER proteins (Warren, 1987; Pelham, 1989). If this is the case the recently identified KDEL receptor (Vaux et al., 1990) should reside in the isolated p53 compartment.

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