p53/58 Binds COPI and Is Required for Selective Transport through the Early Secretory Pathway

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Abstract. p53/58 is a transmembrane protein that continuously recycles between the ER and pre-Golgi intermediates composed of vesicular-tubular clusters (VTCs) found in the cell periphery and at the *cis* face of the Golgi complex. We have generated an antibody that uniquely recognizes the p53/58 cytoplasmic tail. Here we present evidence that this antibody arrests the anterograde transport of vesicular stomatitis virus glycoprotein and leads to the accumulation of p58 in pre-Golgi intermediates. Consistent with a role for the KKXX retrieval motif found at the cytoplasmic carboxyl terminus of p53/58 in retrograde traffic, inhibition of transport through VTCs correlates with the ability of the antibody to block recruitment of COPI coats to the p53/58 cytoplasmic tail and to p53/58-containing membranes. We suggest that p53/58 function may be required for the coupled exchange of COPII for COPI coats during segregation of anterograde and retrograde transported proteins.

Sorting and recycling of proteins during transit through the early secretory pathway (ER, pre-Golgi intermediates, and Golgi compartments) is a critical event required for the selective delivery of cargo to the cell surface (Pelham, 1994; Aridor and Balch, 1996). Segregation is now recognized to include both soluble cargo that contains a carboxyl-terminal retrieval motif (KDEL) and a growing list of transmembrane proteins that possess a di-lysine (KKXX or KXKXX) recycling motif at their cytoplasmic tails (Nilsson et al., 1989; Jackson et al., 1993). The retrograde retrieval of these and other proteins from pre- and *cis*-Golgi elements appears necessary for the maintenance of organelle composition and for the reuse of transport components that participate in vesicle formation and their subsequent fusion to downstream compartments.

Although the precise mechanism of protein recycling in the early secretory pathway is unknown, it is likely to involve the COPI coat complex (coatomer) (Letourneur et al., 1994; Aridor et al., 1995). Yeast mutant strains defective in the COPI subunits α, β′, δ, and ζ are unable to retrieve KKXX-tagged proteins (Letourneur et al., 1994; Cosson et al., 1996; Gaynor and Emr, 1997). This observation is supported by the fact that a glutathione-S-transferase (GST)[1] fusion protein that possesses the carboxyl-terminal KKXX motif binds COPI coat proteins. The binding of coatomer is lost when the di-lysine residues are mutated (Cosson and Letourner, 1994). In addition, a novel di-phenylalanine motif has recently been found to also facilitate binding to COPI, suggesting a bimodal interaction of proteins with coatomer (Fielder et al., 1996; Söhn et al., 1996). Mutations in ARF1, a small GTPase that is essential for COPI recruitment to elements found in the peripheral cytoplasm or at the *cis* face of the Golgi complex (Orci et al., 1993), potently inhibits both ER to Golgi transport and the recycling of KKXX-containing proteins in vivo and in vitro (Dascher and Balch, 1994; Aridor et al., 1995; Tang et al., 1995). While these results emphasize the importance of COPI in retrograde transport, they also suggest that proteins that contain carboxyl-terminal di-lysine/phenylalanine motifs may play a direct role in protein traffic.

Membrane traffic through the exocytic pathway occurs through a selective transport mechanism (Aridor and Balch, 1996). Selective transport is initiated through the formation of COPII-coated vesicles initially discovered by Schekman and colleagues in yeast (Barlowe et al., 1994) and subsequently shown to be essential for ER export in mammalian cells (Kuge et al., 1994; Aridor et al., 1995). COPII coats promote efficient sorting and concentration of cargo during export from the ER (Mizuno and Singer,

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1. *Abbreviations used in this paper*: endo D and H, endoglycosidase D and H; GST, glutathione-S-transferase; NRK, normal rat kidney; VSV-G, vesicular stomatitis glycoprotein; VTC, vesicular tubular cluster.

1993; Balch et al., 1994; Barlowe et al., 1994). After release from the ER, COPII vesicles are targeted to pre-Golgi intermediates composed of vesicular tubular clusters (VTCs). These elements were originally defined by the distribution of the type I transmembrane marker protein p53 in human cell lines (Schweizer et al., 1988) and its homologue, rat p58 (>90% identity) (Saraste et al., 1987; Saraste and Svensson, 1991; Lahtinen et al., 1996), and the small GTPase Rab2 (Chavrier et al., 1990; Tisdale and Balch, 1996). A stereological characterization of the distribution of these intermediates has shown that they are a component of a more complex morphological structure, referred to as "export complexes," in which VTCs are closely juxtaposed to ER membranes that contain numerous COPII budding profiles (Bannykh et al., 1996). Export complexes are found throughout the peripheral cytoplasm but are particularly concentrated at the *cis* face of the Golgi complex through microtubule-dependent events (Presley, J.F., N.B. Cole, and J. Lippincott-Schwartz. 1996. *Mol. Biol. Cell.* 7:74a).

VTCs are the first site of segregation of anterograde and retrograde transported proteins (Aridor et al., 1995; Tang et al., 1995), and the principal site for the recruitment of COPI coats in peripheral sites and in the Golgi region (Lotti et al., 1992; Oprins et al., 1993; Aridor et al., 1995). We have previously demonstrated that a coupled exchange of COPI for COPII coats is essential for the anterograde transport of cargo such as the type 1 transmembrane glycoprotein vesicular stomatitis virus glycoprotein (VSV-G) and the segregation of p58 to recycling vesicles (Aridor et al., 1995). When cells are incubated at reduced temperature (15°C), VTCs accumulate, an event particularly evident in the peripheral regions, and cargo fails to be delivered to the Golgi stack (Saraste and Kuismanen, 1984; Pind et al., 1994; Aridor et al., 1995). These results suggest that a low temperature–sensitive step regulates membrane flow from VTCs to the Golgi region.

The intermediate compartment marker p53 in human cell lines is an unglycosylated, type I transmembrane protein that exists in both dimeric and oligomeric forms (Schweizer et al., 1988), whereas rat p58 is a glycoprotein. p58 is efficiently recruited into COPII-coated vesicles during budding from the ER (Rowe et al., 1996). p53/58 are abundant components of both ER-derived vesicles (Rowe et al., 1996) and isolated VTCs (Schweizer et al., 1990; 1991). The cytoplasmic tails of p53 and p58 are conserved and terminate with the KKXX retrieval motif. This motif, along with additional flanking residues, appears to be critical for the normal recycling itinerary of p53/58 (Itin et al., 1995). Interestingly, p53 has recently been shown to be identical to the mannose-specific membrane lectin, MR60 (Arar et al., 1995), and has been suggested to function as an oligosaccharide-based sorting receptor in the secretory pathway (Itin et al., 1996). However, either a direct role for p53/58 in COPII vesicle budding or COPI binding or its potential role in recycling during ER to Golgi transport has been demonstrated.

To establish whether p58 is required for ER to Golgi transport and to define its potential site of action, we have generated an antipeptide antibody that uniquely recognizes the cytoplasmic tail common to p53 and p58. Here, we report that this antibody potently inhibits the transport of VSV-G from VTCs to the Golgi stack but not export from the ER. Coincident with the block in anterograde transport in the presence of antibody, we observe the accumulation of p58 in VTCs. The block in protein transport directly correlates with inhibition of COPI recruitment to the p53/58 cytoplasmic tail and VTCs in vitro. We propose that p58 and its homologue p53 are coatomer-binding proteins that participate in COPI-coupled segregation events during transport of cargo through VTCs.

Materials and Methods

Materials

Peptides were synthesized and purified, and their structure was confirmed by mass spectroscopy by The Scripps Research Institute Protein/DNA and Mass Spectrometry core facilities (La Jolla, CA). A monoclonal antibody to p53 was a generous gift of H.-P. Hauri (University of Basel, Basel, Switzerland). The clones for monoclonal antibodies M3A5 (Allan and Kreis, 1986) and P5D4 (Kreis, 1986) were kindly provided by T. Kreis (University of Basel, Basel, Switzerland).

Generation of Antipeptide Antibody, Fab Fragments, and Affinity Purification

A peptide that corresponds to the carboxyl-terminal 10 amino acids of p53/58 (QQEAAAKKFF) plus an NH_2-terminal cysteine was synthesized, coupled to maleimide-activated KLH, and used for immunization. The serum was applied to cyanogen bromide–activated Sepharose 4B to which the immunizing peptide was coupled for affinity purification. The column was washed with five bed volumes of PBS, eluted with 0.1 M glycine, pH 2.8, and then neutralized to pH 7.2. The eluate was dialyzed against 25 mM Hepes, pH 7.2, 125 mM KOAc (25/125) and concentrated for use in the semiintact cell transport assay. Fab fragments of affinity-purified anti–cytoplasmic tail were made as described by the manufacturer (Pierce, Rockford, IL).

p53 Cloning and Production of Recombinant p53 Protein

HeLa mRNA was isolated (Oligotex; Qiagen, Chatsworth, CA) and then reverse transcribed for 1 h with AMV Reverse Transcriptase and 1.0 μM specific primer to p53: 5′ GCGGGATCCTACACAAATAGATGAACTACACAGG 3′. The resulting first-strand cDNA was amplified by PCR using the following primers, which generated 5′ NdeI and 3′ BamHI sites: 5′ GGCCATATGGCGGGATCCAGGCAAAGGGGTCTCCGG 3′ and the reverse primer 5′ GCGGGATCCTCAAAAGAATTTTTTGGCAGCTGC 3′. The sequence of the PCR product was confirmed by automated sequencing (Applied Biosystems, Inc., Foster City, CA). The p53 cDNA was cloned into pET3A, and protein was expressed in 100 μM IPTG-induced *Escherichia coli* (BL21) for 3 h at 37°C. The cells were harvested, lysed, Dounce homogenized in 50 mM Tris, pH 8, 200 mM NaCl, 1% Triton X-100, 1 mM EDTA, 40 μg/ml lysozyme, and then centrifuged for 30 min at 15,000 *g*. The soluble fraction was loaded on a 12% SDS-PAGE and the band corresponding to the p53 protein, excised, and electroeluted.

Generation of GST–p53/58 Carboxyl Tail Fusion Protein

Oligonucleotides that corresponded to the cytoplasmic domain of p53/58 (forward primer introduced a BamHI site, 5′ GATCCCAACAAGAAGCAGCTGCAAAA AAATTTTTCTG 3′, reverse primer introduced an EcoRI site, 5′ AATTCAGAAAAATTTTTTTGCAGCTGCTTCTTGTTGG 3′) were synthesized, phosphorylated by T4 polynucleotide kinase, annealed, and the oligonucleotide cassette ligated into pGEX-2T (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). BL21 cells that contained the recombinant pGEX plasmid were grown at 37°C to 0.6_{A600} and then induced with 0.1 mM IPTG for 3 h at 37°C. The liquid culture was centrifuged, and the pellet was resuspended in cold PBS, sonicated, and recentrifuged, and the resulting supernatant was applied to a glutathione Sepharose 4B column (Pharmacia LKB Biotechnology, Inc.). The column was washed with 10 bed volumes of PBS, and the fusion protein was eluted with 5 mM reduced glutathione. The resulting fusion protein was

Table I. ELISA[1] Characterization of Binding of Affinity-purified p53/58 to Peptides

Peptide Sequence	A405
QQEAAAKKFF	365
QQEAAASSFF	249
QQEAAAKKAA	0
AAAKKFF	324
AAASSFF	154
AAASSMP	0
(p23/24c) RRFFKAKKLIE	0
(p53/58) GST-RSQQEAAAKKFF	332
(p53/58) GST-RSQQEAAAKKAA	0
(E3/E19-KKXX) GST-FIDEKKMP	0
(ER protein-KXKXX) GST-KAHKSKTH	0

[1]ELISA performed as described in Materials and Methods.

analyzed by SDS-PAGE and Western blot and was recognized by the antitail antibody.

Analysis of Transport In Vitro

Normal rat kidney (NRK) or CHO clone 15 B cells were infected for 4 h with the temperature-sensitive VSV strain ts045 and then biosynthetically labeled with 100 μCi Trans[^{35}S] for 10 min at the restrictive temperature (39.5°C) to accumulate VSV-G mutant in the ER. The cells were then perforated by swelling and scraping. ER to *cis/medial*-Golgi transport in vitro was measured as described (Davidson and Balch, 1993). Briefly, transport reactions were performed in a final volume of 40 μl in a buffer that contained 25 mM Hepes-KOH, pH 7.2, 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM *N*-acetylglucosamine, an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU rabbit muscle creatine phosphokinase), and 5 μl rat liver cytosol and 5 μl of semiintact cells (~5×10^7 cells/ml, ~25–30 μg total protein) in 50 mM Hepes-KOH, pH 7.2, 90 mM KOAc. To the assay was added the indicated concentration (see Results) of affinity-purified antibody or Fab fragments. The reactions were preincubated on ice for 45 min, subsequently incubated for 90 min at 32°C, and transferred to ice to terminate transport, and the membranes were pelleted, solubilized in buffer, and digested with endoglycosidase H (endo H) (Davidson and Balch, 1993) or endoglycosidase D (endo D) (Beckers et al., 1987) as described. The samples were analyzed by SDS-PAGE and the fraction of VSV-G protein processed to the endo H–resistant or endo D–sensitive forms quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Indirect Immunofluorescence

NRK, BHK, and HeLa cells were plated on coverslips overnight and then fixed in 3% formaldehyde/PBS for 10 min. The fixed cells were then permeabilized with 0.05% saponin in PBS for 10 min, incubated with affinity-purified antipeptide antibody to the cytoplasmic tail of p53/58 for 30 min, washed with PBS, and then stained with antirabbit–conjugated FITC for 30 min. The cells were then washed with PBS, mounted, and viewed under a fluorescence microscope (model Axiovert; Carl Zeiss, Inc., Thornwood, NY). For the morphological assay, NRK cells plated on coverslips were infected with ts045 at 39.5°C for 2–3 h and then shifted to ice and permeabilized with digitonin (20 μg/ml) as outlined previously (Plutner et al., 1992). Coverslips with permeabilized cells were inverted and placed in tissue culture wells that contained the transport cocktail described above, preincubated on ice for 45 min with or without the antibody, and then incubated for 30 min at 32°C. To terminate transport, the cells were transferred to ice and fixed in 3% formaldehyde/PBS for 10 min. Intracellular VSV-G was detected by repermeabilization of the fixed cells with 0.05% saponin in PBS for 10 min, washed with PBS, and then incubated for 30 min with a monoclonal antibody specific for VSV-G protein cytoplasmic tail (P5D4) (Kreis, 1986). Cells were then washed with PBS, costained for 30 min with Texas red anti–mouse antibody, mounted, and viewed as above.

Noninfected digitonin-permeabilized cells were incubated in transport cocktail with or without antibody for 80 min at 15°C to arrest transport in the 15°C pre-Golgi structures. Cells were then either fixed with 3% formaldehyde/PBS or transferred to 37°C for 15 min, fixed, stained with anti–β-COP (M3A5) (Allan and Kreis, 1986), washed, and then costained with anti–mouse Texas red, as described above.

ELISA

Peptides (1 μg/100 μl of 50 mM $NaHCO_3$, pH 9.6) and GST fusion proteins (1 μg/100 μl of 50 mM $NaHCO_3$, pH 9.6) listed in Table I were coated in Nunc-immunomodules at 4°C overnight. The wells were washed in TBS, blocked in TBS/5% FBS for 1 h at 37°C, additionally washed in TBS, then incubated with 1 μg affinity-purified antitail antibody for 3 h at 37°C, washed with TBS, and then incubated with antirabbit–conjugated alkaline phosphatase for 1 h at 37°C. The wells were washed with TBS and then developed with Sigma 104 phosphatase substrate (St. Louis, MO) and read at 405 nm on a microplate reader.

β-COP Binding to GST–p53/58 Cytoplasmic Tail Fusion Protein

GST–p53/58 protein (100 μg) was preincubated with 75 μl of glutathione Sepharose 4B for 4 h at room temperature and then washed three times with 25/125 to remove any unbound protein. Excess antipeptide antibody (100 μg) was added where indicated in the Results and allowed to absorb to the fusion protein for 4 h on ice. Rat liver cytosol (~200 μg total protein in 25/125 [Davidson and Balch, 1993]) was then added and incubated for an additional 4 h at 4°C. The beads were pelleted at 5K for 5 min and the supernatant collected. The beads were then washed four times with 1 ml of 25/125 at 5,000 *g* for 5 min. The resulting supernatants were pooled, precipitated with 20% TCA, and centrifuged, and the pellets were resuspended in sample buffer. The bound rat liver cytosolic proteins were eluted from the fusion protein with 1 ml of 500 mM NaCl in 25/125 after centrifugation at 5K for 5 min. The supernatant was precipitated with 20% TCA and centrifuged, and the pellet was resuspended in sample buffer. All fractions were separated by SDS-PAGE and transferred to nitrocellulose in 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. The membrane was blocked in TBS that contained 5% nonfat dry milk and 0.5% Tween-20, incubated with a monoclonal antibody to β-COP (M3A5), washed, further incubated with a horseradish peroxidase–conjugated anti–mouse antibody, and then developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Determination of COPI Binding to Membranes

NRK cells (two confluent 10-cm dishes) were washed three times with ice-cold PBS. The cells were scraped off the dish with a rubber policeman into 10 mM Hepes, pH 7.2, and 250 mM mannitol and then homogenized with 15 passes of a 27-gauge syringe. The homogenate was pelleted at 500 *g* for 10 min at 4°C, and the supernatant was centrifuged at 16,000 *g* for 20 min at 4°C. The microsomal fraction (pellet) was washed in 1 M KCl in 10 mM Hepes, pH 7.2, for 15 min on ice to remove bound coatomer and then centrifuged at 16,000 *g* for 20 min at 4°C. The membranes were resuspended in 10 mM Hepes, pH 7.2, and 250 mM mannitol and used in the binding reaction as described by Aridor (1995), with modifications. Membrane (30 μg of total protein) was added to a reaction mixture that contained 27.5 mM Hepes, pH 7.2, 2.75 mM MgOAc, 65 mM KOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, and 0.2 U of rabbit muscle creatine kinase. Antibody was added in the amounts indicated in Results, and the reaction mix was incubated on ice for 45 min. Rat liver cytosol (25 μg) and 20 μM GTPγS (to promote constitutive ARF1 activation) were then added and the reactions shifted to 37°C and incubated for 15 min. The binding reaction was terminated by transferring the samples to ice and then adding 1 ml of 25 mM Hepes, pH 7.2, 2.5 mM MgOAC, and KOAc to a final concentration of 250 mM. The samples were vortexed and centrifuged at 16,000 *g* for 10 min at 4°C. The pellet was resuspended in sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, developed, and quantitated by densitometry as described above.

Antibody Neutralization

GST–p53/58 (50 μg in 500 μl of 25/125) or GST (50 μg in 500 μl 25/125) was added to 50 μl of glutathione Sepharose 4B preequilibrated with 25/125 for 4 h at 4°C and then washed three times with 25/125 to remove unbound fusion protein. The beads were resuspended in 18 μl of 25/125 with or without 5 μg of antipeptide antibody, incubated 4 h at 4°C, and centri-

fuged at 5,000 *g* for 5 min, and the supernatants were removed and used in the semiintact cell assay.

Results

An Affinity-purified Antibody to the Carboxyl Terminus of p53/58 Recognizes the Endogenous Protein

To address the potential biochemical role of p53 and p58 in the transport of cargo between the ER and the Golgi, we generated an antipeptide polyclonal antibody that recognizes a cytoplasmic tail peptide (QQEEAAKKFF) common to both proteins (to be referred to as the antitail antibody) (Schweizer et al., 1988; Lahtinen et al., 1996). Affinity-purified antibody detected an ~58-kD protein from NRK and BHK cell lysates on a Western blot (Fig. 1 *A*, lanes *a* and *b*) that comigrated with a protein identified by previously characterized antibodies specific for p58 (Saraste et al., 1987) (Fig. 1 *B*, lanes *a* and *b*). In HeLa cell lysates, the antitail antibody recognized a slightly faster migrating protein of ~53 kD (Fig. 1 *A*, lane *c*), which was also detected by a monoclonal antibody specific for the luminal domain of p53 (Schweizer et al., 1988) (Fig. 1 *B*, lane *c*). This protein is likely to be p53 since the antitail antibody also recognized purified recombinant p53 (Fig. 1 *C*, lane *a*). The antitail antibody did not detect GST fusion proteins (~30 kD) that contained either the KKXX retrieval motif (FIDEKKMP) of the E3/E19 glycoprotein or the KSKXX retrieval motif (KAHKSKTH) of an ER resident protein (Fig. 1 *C*, lanes *b* and *c*, *arrow*). Moreover, the antibody did not detect proteins in cell lysates in the molecular mass range of 24 kD, which have recently been shown to bind coatomer through either KKXX-, KXKXX-, or di-phenylalanine–containing motifs (Fiedler et al., 1996; Söhn et al., 1996). Specifically, the antibody did not detect recombinant gp25l (Waka et al., 1991) or a GST–gp25l tail peptide (KNFFIAKKLV) fusion protein, a representative member of the p24 gene family (Schimmoller et al., 1995; Stamnes et al., 1995; Belden and Barlowe, 1996; Fielder et al., 1996) (not shown). These results suggest that antibody recognition is dependent on an epitope in the carboxyl terminus unique to p58.

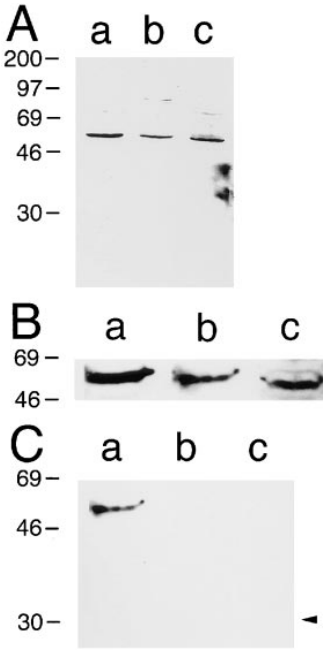

Figure 1. Antibody to the cytoplasmic tail region recognizes p53/58 on Western blots. (*A*) NRK (lane *a*), BHK (lane *b*), and HeLa (lane *c*) cell lysates were separated by SDS-PAGE, transferred to nitrocellulose for Western blot analysis, and probed with affinity-purified anti-p53/58 tail antibody as described in Materials and Methods. (*B*) NRK (lanes *a* and *b*) and HeLa (lane *c*) cell lysates were probed with either antitail antibody (lane *a*), a polyclonal antibody specific for p58 (lane *b*) (Saraste et al., 1987), or with polyclonal antibody to p53 (lane *c*) (Schweizer et al., 1991). (*C*) Purified recombinant p53 (lane *a*), GST-FIDEKKMP (lane *b*) and GST-KAHKSKTH (lane *c*) were probed with antitail antibody on Western blots as described in the Materials and Methods.

To extend these results, an ELISA was performed to define the epitope recognized by the antibody. As shown in Table I, the antibody recognized a peptide that coded for the wild-type p53/58 cytoplasmic tail (QQEAAAKKFF) or a GST fusion protein containing the cytoplasmic tail of p53/58 with similar affinity. Moreover, the GST–peptide fusion proteins containing FIDEKKMP or KAHKSKTH, which were not recognized by antitail antibody on Western blots (Fig. 2 *C*), were similarly unreactive when bound to microtitre wells. Removal of the QQE residues did not affect antibody recognition (Table I), indicating that the epitope responsible for binding was in the terminal seven residues. Mutation of the di-lysine residues to serine had only a partial effect on antibody binding (Table I). However, mutation of the terminal FF residues to either AA or MP completely abrogated antibody recognition (Table I). In contrast, a peptide corresponding to the tail of p23/24c (RRFFKAKKLIE) (Fielder et al., 1996; Söhn et al., 1996), which contains an internal FF motif, was not recognized (Table 1). The combined results indicate that the dominant epitope recognized by the affinity-purified antitail antibody requires terminal FF residues with a weaker contribution of adjacent di-lysine residues. Importantly, neither di-lysine nor internal di-phenylalanine residues are sufficient to elicit antibody recognition.

To confirm that the antibody recognizes p53/58 in vivo, we used indirect immunofluorescence. The affinity-purified antibody labeled clusters of punctate structures largely concentrated near the Golgi complex in HeLa (Fig. 2 *A*) and BHK (Fig. 2 *C*) cells at steady state. In NRK cells (Fig. 2 *B*), numerous punctate structures distributed throughout the cytoplasm characteristic of peripheral pre-Golgi intermediates composed of clusters of vesicular tubular elements (VTCs) were also detected. The labeling of peripheral punctate elements in all cell lines tested were markedly enhanced after incubation at 15°C (not shown), a condition that results in accumulation of p53/58-containing VTCs (Saraste and Svensson, 1991). The distribution observed with the antitail antibody was identical with that reported for antibodies that recognize the luminal domain of p53 in human (Schweizer et al., 1990) and p58 in rat (Saraste and Svensson, 1991) cell lines. These results demonstrate that the antibody detects a protein with the morphological properties of p53/58. All subsequent studies use the affinity-purified antibody for analysis of the role of p53/58 in transport.

Antipeptide Antibody Inhibits ER to Golgi Transport

The antitail antibody was first tested in a semiintact cell assay to evaluate its effect on protein traffic from the ER to the Golgi complex in NRK cells (Davidson and Balch, 1993). This assay makes use of ts045 VSV-G, a protein that has a thermoreversible defect in export from the ER (Lafay, 1974; Plutner et al., 1992). Cells infected for 4 h at the

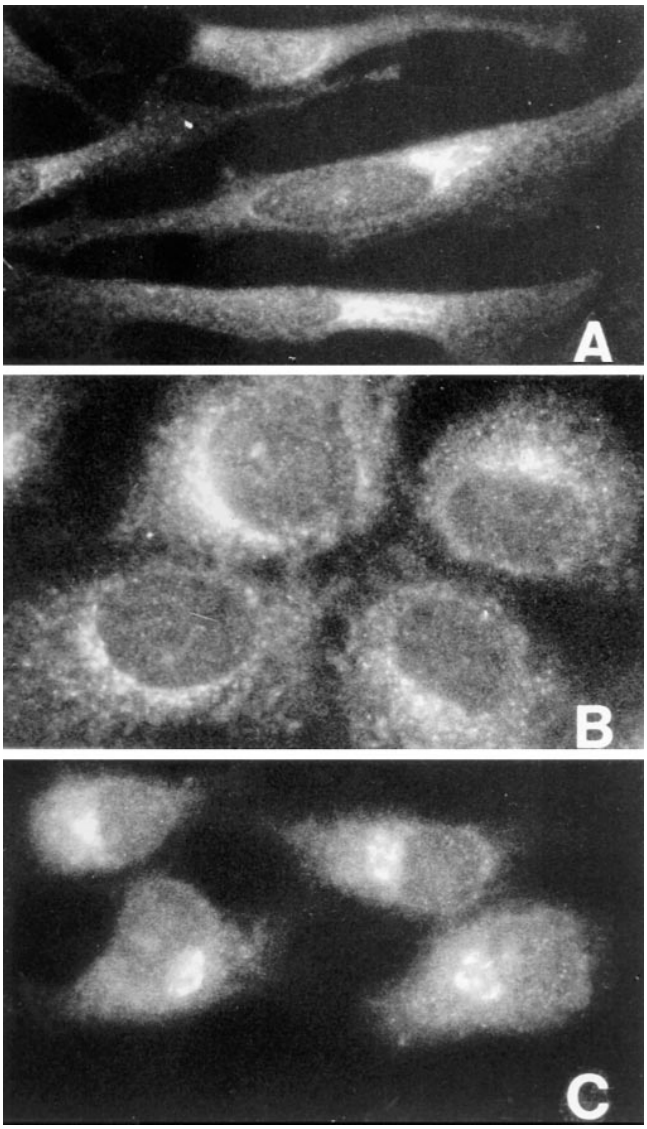

Figure 2. Antipeptide antibody identifies a protein with a p53/58-like distribution localized to VTCs in peripheral and Golgi adjacent sites. (*A*) HeLa, (*B*) NRK, and (*C*) BHK cells were plated on coverslips and cultured overnight. The cells were fixed, permeabilized, and stained with affinity-purified polyclonal antibody to the cytoplasmic domain of p53/58, followed by FITC-goat anti–rabbit as described in Materials and Methods. In all three cell types, punctate elements near the Golgi complex were labeled by the antibody. This staining pattern is similar to that observed with antibodies that recognize the luminal domain of p53 and p58 (Schweizer et al., 1990; Saraste and Svensson, 1991).

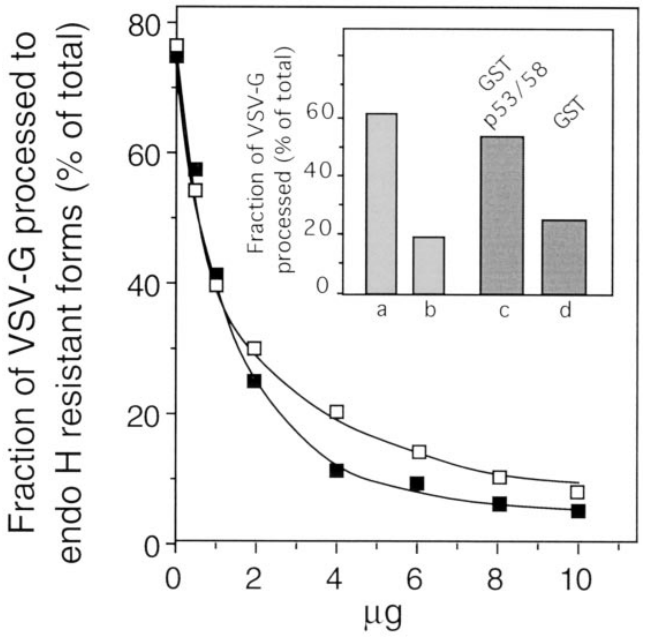

Figure 3. Antibody to the p53/58 carboxyl tail inhibits transport from the ER to the Golgi complex. (*A*) Semiintact NRK cells were prepared and incubated in vitro as described in Materials and Methods. Semiintact cells were incubated with the indicated concentration of antibody (*closed squares*) or Fab fragments (*open squares*) for 45 min on ice and then transferred to 32°C for 90 min. (*Inset*) Preabsorption of the antibody to the cytoplasmic tail neutralizes its inhibitory property. Semiintact cells were incubated in the absence (*a*) or presence of 5 μg of antipeptide antibody (*b–d*) and pretreated as follows: (*b*) no pretreatment; (*c*) antibody preincubated with a GST–p53/58 tail fusion protein bound to glutathione Sepharose 4B beads; (*d*) antibody preincubated with GST-glutathione Sepharose 4B beads as described in Materials and Methods. In *c* and *d*, the unbound fraction was added to the transport assay.

restrictive temperature (39.5°C) to retain VSV-G in the ER (Plutner et al., 1992; Balch et al., 1994) were rapidly transferred to ice and perforated to generate semiintact cells (Beckers et al., 1987). Export of VSV-G from the ER was initiated by incubation of perforated cells at the permissive temperature (32°C) in the presence of cytosol and ATP (Davidson and Balch, 1993). The assay measures transport of the VSV-G protein to the Golgi stack by following the conversion of its two N-linked oligosaccharides from the endo H–sensitive oligosaccharide form found in the ER to the endo H–resistant species found in the *cis/medial* region of the Golgi stack (Schwaninger et al., 1991; Davidson and Balch, 1993).

Preincubation of semiintact NRK cells with affinity-purified antibody led to a dose-dependent inhibition of ER to Golgi transport (Fig. 3). The processing of VSV-G to endo H–resistant forms was reduced by 50% in the presence of ~2 μg antibody with complete inhibition at 8–10 μg (Fig. 3, *closed squares*). This inhibition was not a consequence of protein aggregation as Fab fragments also inhibited acquisition of endo H resistance at a level comparable to that of the intact antibody (Fig. 3, *open squares*). To provide further proof that the block in transport was due to the specific neutralization of a p53/58 carboxyl-terminal epitope, antibody was incubated with a GST–p53/58 carboxyl tail fusion protein (GST–p53/58 tail) bound to glutathione Sepharose 4B. The resulting nonabsorbed fraction was tested for activity in the semiintact cell assay. Preincubation of the antibody with the GST–p53/58 tail beads efficiently neutralized its inhibitory effect on protein traffic (Fig. 3, *inset*, *c*). In contrast, inhibition was not affected by incubation of antibody with control GST beads that lacked the p53/58 tail (Fig. 3, *inset*, *d*). These results show that the antibody blocks transport in NRK cells through a specific interaction with p58 and is the first dem-

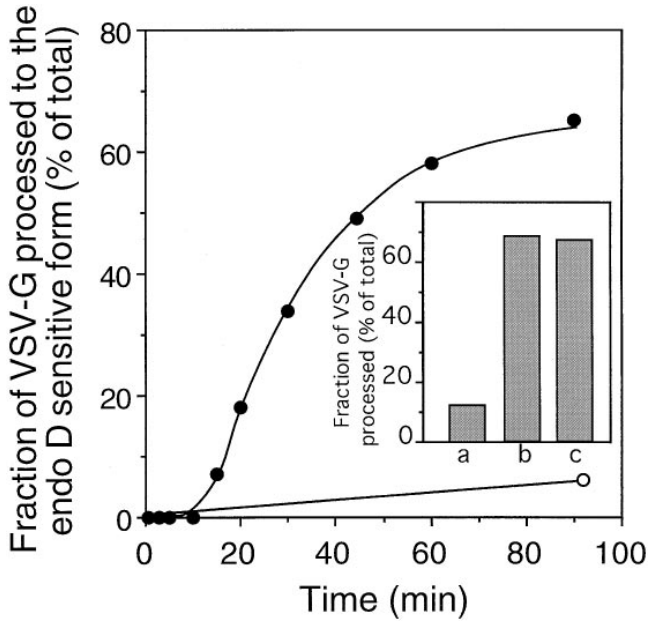

Figure 4. The antitail antibody blocks transport before the delivery of cargo to the Golgi compartment containing α-1,2 mannosidase I. Semiintact CHO clone 15B cells were incubated at 32°C in a complete transport cocktail for indicated time in the absence (*closed circles*) or presence (*open circles*) of antitail antibody. The amount of VSV-G processed to endo D–sensitive forms was determined as described in Materials and Methods. (*Inset*) Antibody inhibition precedes the Ca^{2+}-dependent fusion of VTCs to the Golgi stack. Semiintact CHO clone 15B cells were incubated in a transport cocktail that contained 5 mM EGTA for 60 min at 32°C to accumulate VSV-G in post-ER, pre-Golgi VTCs (Pind et al., 1994). The cells were pelleted, resuspended in a transport cocktail that contained either 5 mM EGTA (*a*), 0.1 μM Ca^{2+} (*b*), or 0.1 μM Ca^{2+} and 10 μg of antitail antibody (*c*), and incubated at 32°C for 90 min. The fraction of VSV-G processed to the endo D–sensitive form was determined as described in Materials and Methods.

onstration that p58 may be required for ER to Golgi transport.

Antibody to the p53/58 Cytoplasmic Tail Inhibits Transport of VSV-G From VTCs to the Golgi Stack

To localize the step in transport sensitive to antibody, we made use of the CHO clone 15B cell line, which lacks the *cis/medial*-Golgi enzyme *N*-acetylglucosamine-transferase I (GlcNAC Tr I) (Tabas and Kornfeld, 1979). In 15B cells, processing of VSV-G oligosaccharides does not proceed beyond the Man_5 form, which appears in response to trimming by α-mannosidase I found in the *cis* region of Golgi stack (Beckers et al., 1987). The Man_5 form of VSV-G is uniquely susceptible to digestion with endo D (Beckers et al., 1987), providing a direct measure for VSV-G delivery to the *cis*-most face of the Golgi. As shown in Fig. 4 (*closed circles*), semiintact CHO 15B cells incubated in the absence of antibody at 32°C processed VSV-G to the Man_5 form. In contrast, in the presence of antibody, processing was completely inhibited (Fig. 4, *open circles*).

Since the above results demonstrated that VSV-G transport was blocked at a pre-Golgi step, we next determined whether antibody inhibited a Ca^{2+}-dependent step involved in the delivery of cargo from pre-Golgi VTCs to Golgi compartments (Beckers and Balch, 1989; Pind et al., 1994; Aridor et al., 1995). This step has been previously well characterized in both yeast (Rexach and Schekman, 1991) and mammalian cells (Beckers and Balch, 1989; Pind et al., 1994; Aridor et al., 1995). Using immunoelectron microscopy, we have demonstrated that Ca^{2+} depletion prevents a late fusion event, resulting in the accumulation of VSV-G–containing VTCs (Pind et al., 1994). Semiintact 15B CHO cells were incubated in a transport cocktail containing 5 mM EGTA for 60 min at 32°C to accumulate VSV-G in VTCs. Cells were then pelleted, held on ice in a Ca^{2+}-containing transport cocktail in either the absence or presence of antibody, and subsequently incubated at 32°C for an additional 90 min. In both control (Fig. 4, *inset*, *b*) and antibody-treated cells (Fig. 4, *inset*, *c*), VSV-G was efficiently processed to the endo D–sensitive form. Thus, the site of antibody action is before the Ca^{2+}-dependent fusion of VTCs to the Golgi stack.

We have shown previously that VSV-G exits the ER via COPII-coated vesicles and accumulates in VTCs when cells are incubated at 15°C (15°C-VTCs) in vitro (Aridor et al., 1995). To determine if the antibody inhibits transport of VSV-G from 15°C-VTCs to the Golgi stack, semiintact cells were incubated at reduced temperature for 80 min in a complete transport cocktail (Fig. 5, *circles*) and then transferred to 32°C and at the indicated time (*Δt*) either shifted to ice (Fig. 5, *closed circles*) or supplemented

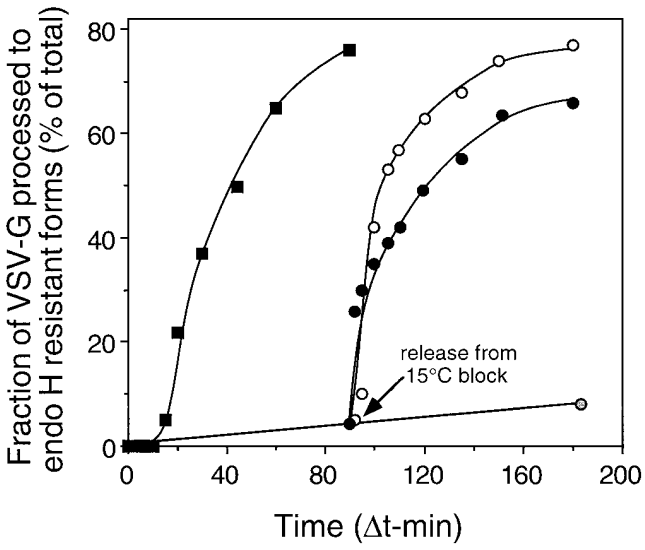

Figure 5. Antibody inhibits transport of VSV-G from 15°C-VTCs to the Golgi stack. Semiintact NRK cells were either incubated in a transport cocktail for the indicated time at 32°C (*closed squares*) or preincubated at reduced temperature (15°C) for 80 min to accumulate VSV-G in pre-Golgi VTCs (*circles*). Cells preincubated at 15°C were either maintained at 15°C (*gray circle*) or shifted to 32°C and incubated for the indicated time (*Δt*) and then transferred to ice to terminate transport (*closed circles*), or supplemented with 10 μg antipeptide antibody at the indicated time (*Δt*) (*open circles*) and incubated for a total of 90 min. The fraction of VSV-G processed to the endo H–resistant forms was determined as described in Materials and Methods.

with antibody and incubated for a total time of 90 min (Fig. 5, *open circles*). Cells incubated continuously at 15°C did not acquire endo H resistance, which indicates minimal leakage from VTCs to the Golgi over the 190-min time course (Fig. 5, *gray circle*). Control cells transferred from 15 to 32°C rapidly processed VSV-G to endo H–resistant forms (Fig. 5, *closed circles*). In this case, the 10–15-min lag period typically observed after export of VSV-G from the ER upon shift from 39.5 to 32°C (Fig. 5, *closed squares*) was completely absent (Fig. 5, *closed circles*), attesting to the prior accumulation of VSV-G in post-ER intermediates at reduced temperature. Intriguingly, semiintact cells treated with antibody before shift from 15 to 32°C failed to process VSV-G to endo H–resistant forms (Fig. 5, *open circle*, *arrow*). However, this sensitivity was rapidly lost. Incubation of cells at 32°C for as little as 2 min before the addition of antibody led to >60% of the total VSV-G migrating to an antibody-insensitive step. These results demonstrate that the function of p58 cannot be fulfilled in cells incubated at 15°C. However, the rapid migration of VSV-G through the antibody-sensitive step reveals that the activity of p58 is linked to an early step in VTC function during recovery at 32°C.

VSV-G Accumulates in VTCs in the Presence of the Antitail Antibody

Although it was clear that p58 was required for the transit of VSV-G from VTCs to the Golgi stack, it remained possible that the protein was also essential for export from the ER given its lectin-like properties (Arar et al., 1995; Itin et al., 1996). To address this issue, we used a morphological assay in which NRK cells were infected with ts045 VSV-G for 3 h at 39.5°C (to restrict VSV-G to the ER) (Fig. 6 *A*) (Plutner et al., 1992). After permeabilization, cells were incubated in the absence or presence of antibody for 45 min on ice and then transferred to 32°C for 30 min, and the distribution of VSV-G was determined by indirect immunofluorescence. Control cells incubated at 32°C in the absence of antibody efficiently transported VSV-G to the juxtanuclear Golgi complex (Fig. 6 *C*). This distribution overlapped with the typical steady-state distribution of p58 in VTCs localized predominantly to the *cis*-Golgi region (Fig. 6, *B* and *D*). In contrast, permeabilized cells incubated in the presence of antibody largely accumulated VSV-G in numerous punctate VTCs scattered throughout the perinuclear and peripheral cytoplasm (Fig. 6 *E*). VSV-G present in peripheral punctate elements completely overlapped with that of p58 (Fig. 6 *F*). The antibody had no effect on the distribution of the Golgi complex as assessed by staining with *Lens culinaris*, which recognizes *cis*/*medial*-Golgi compartments (not shown) (Liener et al., 1986; Tisdale et al., 1992). Moreover, cells incubated at 15°C for 80 min in the presence of antibody accumulated VSV-G in pre-Golgi VTCs (not shown). These results demonstrate that the antibody did not affect export in a manner similar to that of the Sar1 GDP-restricted mutant, which prevents COPII assembly and blocks exit of VSV-G from the ER (Kuge et al., 1994; Aridor et al., 1995), but interfered specifically with transit from VTCs to compartments of the Golgi stack. These results are consistent with the lack of processing of VSV-G to endo D– (Fig. 3) and endo H– (Fig. 4) resistant forms in the presence of antibody.

The fact that the antibody caused retention of VSV-G in punctate VTCs, which also strongly labeled for p58, prompted us to examine if antibody-treated cells were altered in their ability to transport p58 from these intermediates to the more typical Golgi-like steady-state distribution observed at 32°C (Fig. 6 *B*). Uninfected, permeabilized NRK cells were incubated in the absence (Fig. 7, *A* and *B*) or presence (Fig. 7 *C*) of antibody at 15°C for 80 min to accumulate 15°C-VTCs (Aridor et al., 1995). Cells were subsequently shifted to 32°C for 20 min. In the absence of antibody, p58 redistributed from its punctate distribution in pre-Golgi intermediates (Fig. 7 *A*) to the typical perinuclear, steady-state distribution (Fig. 7 *B*) found before incubation at reduced temperature (Fig. 6 *B*). In contrast, antibody-treated cells retained p58 in numerous, peripheral punctate elements (Fig. 7 *C*), a distribution very similar to that observed at reduced temperature (Fig. 7 *A*). This result is identical to the effect of ARF1 mutants that interfere with coatomer function (Dascher and Balch, 1994; Aridor et al., 1995). The apparent inability of both VSV-G and p58 to be mobilized from VTCs raises the possibility that the antitail antibody coordinately interferes with the transit of both anterograde and retrograde transported proteins.

Antibody to the p53/58 Cytoplasmic Domain Blocks Recruitment of COPI

Segregation of p58 from VSV-G during transit through VTCs requires COPI (Aridor et al., 1995). Because COPI components bind to carboxyl-terminal di-lysine and diphenylalanine motifs (Cosson and Letourner, 1994; Fiedler and Simons, 1995; Söhn et al., 1996), the presence of these residues at the cytoplasmic tail of p53/58 suggested that the antibody may inhibit transport by interfering with the binding of coatomer. To address this question morphologically, uninfected, permeabilized NRK cells were preincubated at 15°C for 80 min in the absence of antibody to accumulate VTCs containing p58 (as shown in Fig. 7 *A*). The cells were then incubated at 37°C for 20 min in the absence (Fig. 8 *A*) or presence (Fig. 8 *B*) of antibody. Control cells (lacking antibody) showed a typical intense staining for β-COP in the Golgi region (Duden et al., 1991; Oprins et al., 1993; Aridor et al., 1995). In contrast, cells incubated in the presence of antibody displayed a striking reduction in the number of β-COP–positive structures (Fig. 8 *C*). A similar reduction in coatomer recruitment in the presence of antibody was observed by incubating cells at 32°C for 30 min without the 15°C preincubation (not shown). The loss of β-COP–positive elements was not observed using a number of other antibodies, including several monoclonal reagents specific for the small GTPases Rab1 (Plutner et al., 1991; Saraste et al., 1995) and Rab2 (Chavrier et al., 1990), which are localized to pre-Golgi intermediates. Moreover, in the case of infected cells, a polyclonal specific to the cytoplasmic tail of VSV-G protein had no effect on the distribution of β-COP–positive elements (not shown). Thus, the anti-p53/58 tail antibody appears to significantly modify, either directly or indirectly,

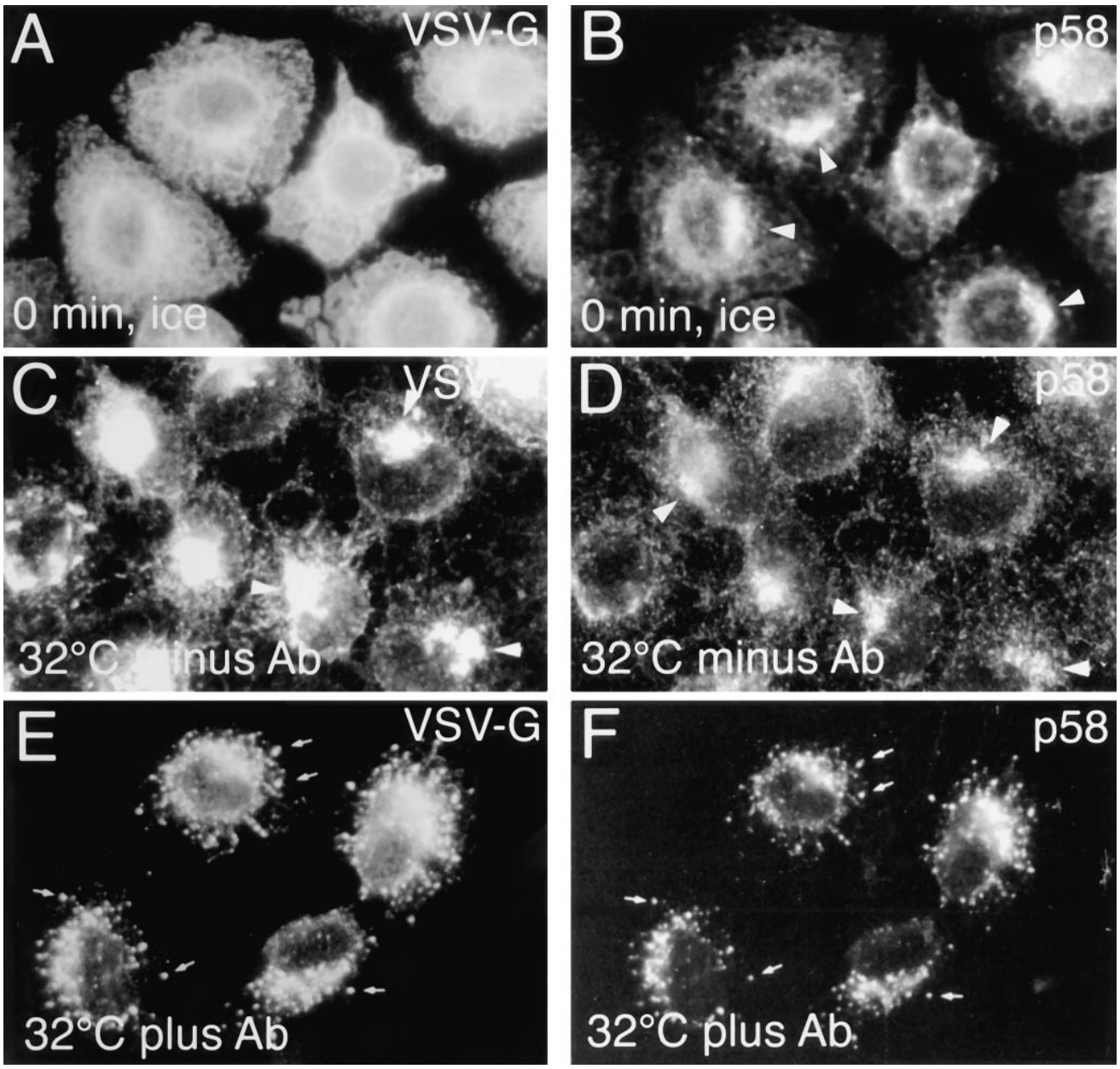

Figure 6. VSV-G protein accumulates in VTCs in the presence of antibody. NRK cells grown on coverslips were infected with ts045 VSV for 3 h at 39.5°C to accumulate ts045 VSV-G in the ER (Plutner et al., 1992; Balch et al., 1994). The cells were shifted to ice, permeabilized as described in Materials and Methods, and then preincubated in a complete transport cocktail for 45 min on ice in the absence (*A–D*) or presence of 10 μg of antitail antibody (*E* and *F*). Subsequently, the cells were then either retained on ice (*A* and *B*) or shifted to 32°C for 30 min (*C–F*) and were transferred to ice to terminate transport and the distribution of VSV-G (*A*, *C*, and *E*) and p58 (*B*, *D*, and *F*) determined by indirect immunofluorescence as described in Materials and Methods. In control cells (*C* and *D*), VSV-G was transported to the perinuclear Golgi region that partially overlaps with the distribution of p58 (*arrowheads*). Antibody treatment resulted in the accumulation of VSV-G in punctate VTCs containing p58 (*E* [VSV-G] and *F* [p58], *arrows*).

the ability of VTCs located in peripheral and Golgi adjacent sites to recruit COPI.

Antitail Antibody Inhibits the Binding of COPI to the GST–Tail Fusion Protein and to Microsomes

To assess the possible effect of antibody on coatomer recruitment by p53/58, we analyzed COPI binding to the GST fusion protein containing the carboxyl terminus QQEEAAKKFF residues bound to glutathione Sepharose 4B beads (GST–tail beads). GST–tail beads were incubated with rat liver cytosol, which serves as a rich source of coatomer. After incubation, the beads were washed extensively with either a low (75 mM) or high (500 mM) salt-containing buffer, and the unbound (low salt wash) and bound-released protein (high salt wash) were analyzed by SDS-PAGE and Western blotting for β-COP. Control beads containing the GST construct alone (minus tail) did

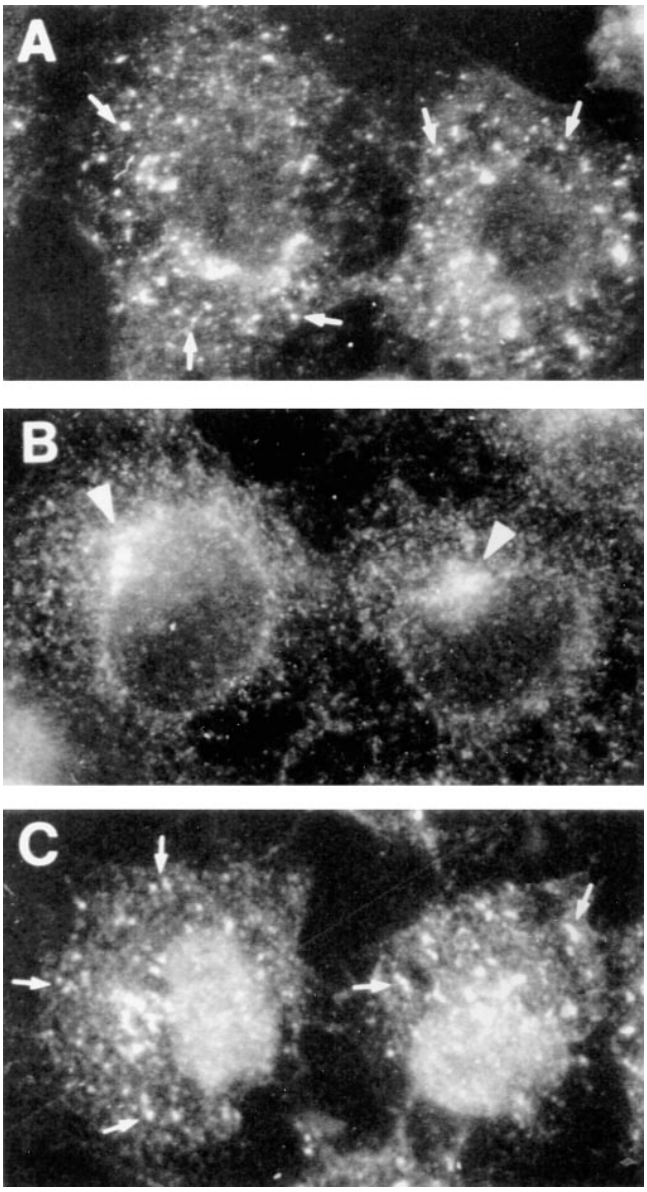

Figure 7. Antitail antibody inhibits the movement of p58 from VTCs. Uninfected NRK cells grown on coverslips were permeabilized as described and incubated in the absence (*A* and *B*) or presence (*C*) of antibody for 80 min at 15°C. The cells were then either retained on ice (*A*) or shifted to 37°C for 20 min (*B* and *C*). Cells were transferred to ice to terminate transport, and the distribution of p58 was determined by indirect immunofluorescence. Arrows denote typical VTCs that label for VSV-G. Arrowheads denote VTCs in the central Golgi region at steady state.

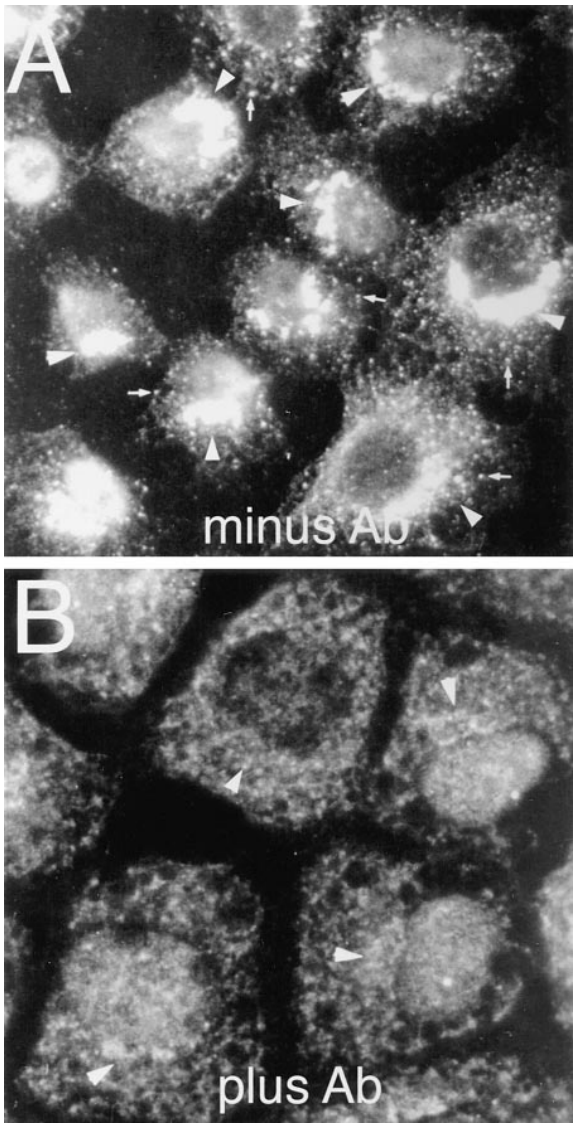

Figure 8. Antitail antibody prevents the recruitment of β-COP to membranes. Uninfected NRK cells grown on coverslips were permeabilized as described (Plutner et al., 1992), incubated at reduced temperature for 80 min at 15°C, and then transferred to 37°C for 20 min in the absence (*A*) or presence (*B*) of antitail antibody, and the distribution of β-COP was determined as described in Materials and Methods.

not retain β-COP after the high-salt wash (Fig. 9 *A*, lanes *a–c*). In contrast, GST–tail beads retained β-COP (Fig. 9 *A*, lane *f*). No binding was detected to GST–tail beads in which the di-lysine motif in the carboxyl-tail was mutated to serine residues (not shown). Importantly, preabsorption of the GST–tail beads with the antitail antibody completely blocked β-COP binding (Fig. 9 *A*, lane *i*), consistent with the observation that the antibody blocks the recruitment of COPI to membranes in vivo (Fig. 8).

To examine whether we could detect a similar effect of the antitail antibody on the recruitment of COPI to VTCs and early Golgi compartments in vitro, membranes were prepared from whole cell homogenates, pretreated with a high-salt wash to remove the loosely bound coatomer, and incubated at 37°C in the presence of cytosol, ATP, and GTPγS, a nonhydrolyzable analog of GTP that constitutively activates the small GTPase ARF1 involved in coatomer binding to VTCs (Aridor et al., 1995). Previous studies have demonstrated that coatomer present on Golgi membranes actively involved in COPI vesicle formation is resistant to a high-salt wash (500 mM), whereas inactive forms can be readily removed by a low (75 mM) salt wash (Aridor et al., 1995; Ostermann et al., 1993; Stamnes and Rothman, 1993). In the absence of antibody, GTPγS

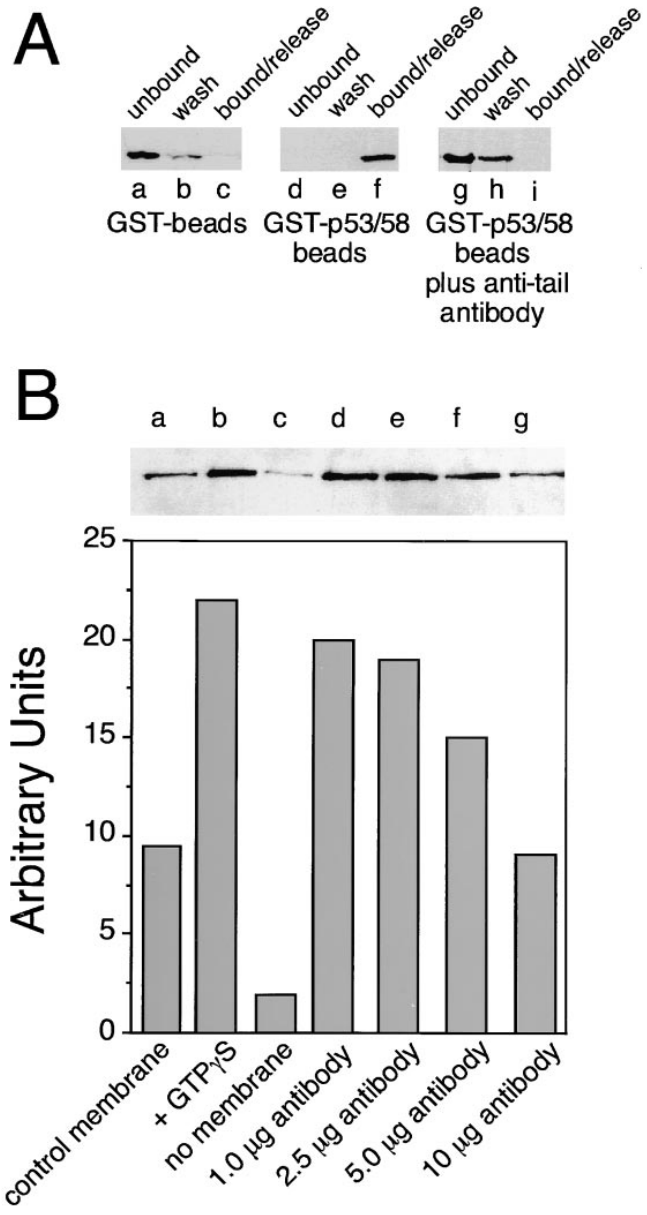

Figure 9. Antitail antibody blocks binding of β-COP to a GST fusion protein containing the carboxyl terminus of p53/58 and prevents the recruitment of COPI coats to microsomes. (*A*) GST (lanes *a–c*) or a GST fusion protein containing the carboxyl terminus of p53/58 (lanes *d–i*) was bound to glutathione Sepharose 4B beads and preincubated in the absence (lanes *a–f*) or presence (lanes *g–i*) of antipeptide antibody as described in Materials and Methods. Rat liver cytosol was then added and incubated for an additional 4 h at 4°C. In each case, the unbound fraction (lanes *a*, *d*, and *g*), the low (75 mM) salt wash (lanes *b*, *e*, and *h*), and the high (500 mM) salt wash (lanes *c*, *f*, and *i*) were prepared as described in Materials and Methods. The amount of β-COP in each fraction was determined by Western blotting and densitometry. (*B*) Microsomes were prepared from whole cell homogenates as described in Materials and Methods, mixed with cytosol and 20 μM GTPγS, and either not incubated (lane *a*) or incubated for 15 min at 37°C (lanes *b–g*) in the absence (lanes *a–c*) or presence (lanes *d–g*) of the indicated amount of antitail antibody. Membranes were transferred to ice and pelleted, and the amount of β-COP (arbitrary units) was determined by Western blotting and densitometry. In lane *c*, membranes were omitted from the cocktail.

markedly stimulated (~2.5-fold) the level of the high salt resistant form of COPI bound to microsomes during a brief (15 min) incubation at 37°C (Fig. 9 *B*, compare lane *a* [ice] to lane *b* [15 min, 37°]). COPI appearing in the high-speed pellet after the high-salt wash was membrane dependent, as little β-COP was detected when GTPγS was incubated with cytosol alone (Fig. 9 *B*, lane *c*). Addition of antibody led to a dose-dependent inhibition of GTPγS-induced coatomer binding to membranes (Fig. 9 *B*, lanes *d–g*) to levels observed to the control level before incubation (Fig. 9 *B*, lane *a*). The amount of antibody required to block β-COP binding to membranes was comparable to that required to significantly inhibit protein transport in semiintact cells (Fig. 3). No effect on coatomer binding to membranes prepared from noninfected cells was observed with either Rab1- or Rab2-specific antibody (not shown). The effect of the p53/58 tail antibody on β-COP recruitment supports the conclusion that displacement of COPI from VTCs interferes with vesicular traffic and that p58 may participate at this step in such events.

Discussion

We have generated and characterized a polyclonal antibody that recognizes the cytoplasmic tail of p53/58. Affinity-purified antibody detected a single protein in cell lysates of NRK and BHK cells that comigrated with the p58 protein recognized by a previously characterized p58-specific polyclonal antibody (Saraste et al., 1987; Saraste and Svensson, 1991), and a slightly lower molecular weight species detected by a p53-specific monoclonal antibody in HeLa cells (Schweizer et al., 1988). Indirect immunofluorescence showed that the anti-p53/58 tail antibody stained vesicular tubular structures located near the Golgi complex in multiple cell types. An identical pattern has been observed for antibodies that bind to the luminal domains of p53 in human (Schweizer et al., 1988) and p58 (Saraste and Svensson, 1991) in rat cell lines. Although the cytoplasmic domain of p53/58 contains a terminal di-lysine motif, GST fusion proteins that terminate with other KKXX or KXKXX retrieval motifs were not recognized by antibody. Notably, we were unable to detect binding to a GST fusion protein containing the cytoplasmic tail of the p24 family member gp25l, nor were we able to detect binding of antibody to proteins in the 20–25-kD molecular mass range on immunoblots. This region of the gel would be expected to include members of the p24 gene family, which frequently contain coatomer binding motifs (Schimmoller et al., 1995; Stamnes et al., 1995; Fielder et al., 1996; Söhn et al., 1996). Epitope mapping led us to conclude that antibody primarily detects the terminal di-phenylalanine residues in the context of adjacent di-lysine residues. The combined results conclusively demonstrate that the epitope recognized by the antipeptide antibody is specific for residues found in the p53/58 cytoplasmic tail and not other coatomer-binding proteins identified to date.

Using the antitail antibody, we have provided the first evidence that p53/p58, a protein found in ER-derived vesicular carriers (Rowe et al., 1996) and abundant in VTCs (Schweizer et al., 1991), is likely to play an important role in the transport of cargo from the ER to the Golgi com-

plex. Incubation in the presence of antibody or Fab fragments arrested both the anterograde transport of VSV-G from VTCs to the Golgi stack and the recycling of p58. This result is entirely consistent with previous observations in which VTCs were found to be the first site of segregation of VSV-G and p58 (Aridor et al., 1995; Tang et al., 1995) and that this sorting event involves a coupling between the disassembly of COPII coats and the assembly of COPI coat (Aridor et al., 1995; Rowe et al., 1996). Although a recent report concluded that the di-phenylalanine motif present in the cytoplasmic tail of p24 family members was required for ER export (Fielder et al., 1996), the rate of appearance of early Golgi processed forms of a CD8-p24 tail peptide chimera is more consistent with previous results that have demonstrated that FF residues do not affect ER export, rather the efficiency of retrieval of p53 (Itin et al., 1995). As such, these studies provide a separate line of evidence that the residues recognized by the antitail antibody modulate the efficiency of coatomer interaction during recycling.

While we were able to document rigorously that the antitail antibody blocks VSV-G transport to the Golgi, its effects on p58 recycling to the ER relied on our observation using indirect immunofluorescence, which showed that p58 was retained and/or accumulated in VTCs in the presence of antitail antibody. This result is very similar to the effect of ARF1 mutants that prevent proper coatomer function (Dascher and Balch, 1994; Aridor et al., 1995; Rowe et al., 1996). The ability of the antibody to block recycling of p53/58 will need to be analyzed more rigorously using biochemical assays that measure retrograde transport of p53/58 to the ER.

The site of p53/58 function inhibited by the antitail antibody was established by a combination of biochemical and morphological approaches. VTCs are dynamic structures that undergo continuous maturation (Aridor et al., 1995) and translocation via microtubules to the central Golgi region (Presley, J.F., N.B. Cole, and J. Lippincott-Schwartz. 1996. *Mol. Biol. Cell.* 7:74a). We have recently shown that VTCs are components of a more elaborate structure, referred to as "export complexes" (Bannykh et al., 1996), which contain numerous COPII budding profiles facing a central cavity housing COPI coated VTCs. VTCs accumulate at reduced temperature (15°C) and contain enhanced levels of p53/58. Although the antitail antibody blocked transport of VSV-G from 15°C-VTCs to the Golgi stack, this inhibition was lost after a brief shift from 15 to 32°C or after maturation to a later step by incubation in the absence of Ca^{2+} (Pind et al., 1994). Thus, at least one aspect of p53/58 function, as measured by the effects of antibody binding to its carboxyl tail, appears during the temporally restricted step associated with the segregation of p53/58 from cargo during transit through VTCs (Aridor et al., 1995; Tang et al., 1995).

Although the precise role of VTCs in mediating segregation of anterograde and retrograde transported protein is unknown, we have provided a new line of evidence that it may involve p58 in its capacity to recruit COPI. This interpretation is consistent with the presence of the KKXX motif at the cytoplasmic tail of p53/58, a motif that has been previously demonstrated to be involved in COPI binding (Cosson and Letourner, 1994) and in retrograde transport (Jackson et al., 1993; Letourneur et al., 1994). Three lines of evidence support the importance of coatomer binding to p53/58 during transit through VTCs: (*a*) The binding of COPI to the GST–p53/58 tail fusion protein was sensitive to the antitail antibody; (*b*) the antibody markedly reduced binding of coatomer to microsomes in vitro; and (*c*) β-COP labeling of pre-Golgi intermediates in the peripheral and perinuclear region of permeabilized cells was greatly reduced in the presence of antibody. The studies on COPI recruitment used the intact polyclonal reagent and therefore could potentially have unanticipated indirect effects. However, we have observed potent inhibition of transport with Fab's suggestive of a direct effect, and our results are consistent with previous observations that both brefeldin A and a trans dominant mutant of ARF1 restricted to the GDP-bound form also inhibit COPI recruitment to VTCs, and in so doing, block both anterograde and retrograde transport (Aridor et al., 1995).

The above results raise the surprising possibility that p53/58 plays an important role, either directly or indirectly, in regulating COPI binding to VTCs and early Golgi compartments. Consistent with a direct role is the observation that p53/58 is a major protein constituent of pre-Golgi intermediates (Schweizer et al., 1990, 1991). However, p53/58 is not unique in its ability to bind coatomer, as a number of other proteins contain KKXX, KXKXX, or internal FF residues that can potentially function in recruitment. At least two members of the p24 gene family appear to be components of COPII carrier vesicles—others have been shown to be associated with purified COPI vesicles (Stamnes et al., 1995; Elrod-Erickson and Kaiser, 1996; Fielder et al., 1996; Söhn et al., 1996). Because many of these proteins may actively recycle, the marked decrease in COPI association with membranes observed in vitro in response to the p53/58 antitail antibody may be, in part, due to a general block in the retrograde pathway involving these other coatomer-binding proteins. In addition, the observation that the antibody blocks stable (high salt resistant) binding of COPI coats to microsomal membranes may reflect the possibility that coatomer recruitment is a combinatorial event requiring more than one protein (Fielder et al., 1996) and that the antibody augments this dependency.

Although the antitail antibody potently blocked transit of VSV-G through VTCs, it did not block export of VSV-G from the ER. It has recently been proposed that p53/58 serves as a lectin for the sorting of cargo from resident ER proteins (Itin et al., 1995, 1996). Consistent with this possibility is our previous observation that p53/58 is a component of ER-derived vesicular COPII carriers containing VSV-G (Rowe et al., 1996). p53/58 may use its luminal lectin-binding domain for protein export and its cytosolic domain for retrieval. In general, our results reinforce a model (Aridor et al., 1995; Rowe et al., 1996) in which a critical step in the segregation of anterograde and retrograde transported protein through VTCs is related to the recruitment of COPI, a step possibly involving the function of p53/58.

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References

Allan, V.J., and T.E. Kreis. 1986. A microtubule-binding protein associated with membranes of the Golgi apparatus. *J. Cell Biol.* 103:2229–2239.

Arar, C., V. Carpentier, J.-P. Le Caer, M. Monsigny, A. Legrand, and A.-C. Roche. 1995. ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. *J. Biol. Chem.* 270:3551–3553.

Aridor, M., and W.E. Balch. 1996. Principles of selective transport: coat components hold the key. *Trends Cell Biol.* 6:315–320.

Aridor, M., S. Bannykh, T. Rowe, and W.E. Balch. 1995. Sequential coupling between COPII and COPI coats in endoplasmic reticulum to Golgi transport. *J. Cell Biol.* 131:875–893.

Balch, W.E., J.M. McCaffery, H. Plutner, and M.G. Farquhar. 1994. Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell.* 76:841–852.

Bannykh, S., T. Rowe, and W.E. Balch. 1996. The organization of endoplasmic reticulum export complexes. *J. Cell Biol.* 135:19–35.

Barlowe, C., L. Orci, T. Yeung, M. Hosobuchi, S. Hamamoto, N. Salama, M.F. Rexach, M. Ravazzola, M. Amherdt, and R. Schekman. 1994. COPII: a membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell.* 77:895–907.

Beckers, C.J.M., and W.E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245–1256.

Beckers, C.J.M., D.S. Keller, and W.E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–534.

Belden, W.J., and C. Barlowe. 1996. Erv25p, a component of COPII-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. *J. Biol. Chem.* 271:26939–26946.

Chavrier, P., R.G. Parton, H.P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell.* 62;317–329.

Cosson, P., and F. Letourneur. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science (Wash. DC).* 263:1629–1631.

Cosson, P., C. Demolliere, S. Hennecke, R. Duden, and F. Letourneur. 1996. d- and z-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. *EMBO (Eur. Mol. Biol. Organ.) J.* 15: 1792–1798.

Dascher, C., and W.E. Balch. 1994. Dominant inhibitory mutants of ARF1 inhibit ER to Golgi transport and trigger the disassembly of the Golgi apparatus. *J. Biol. Chem.* 269:1437–1448.

Davidson, H.W., and W.E. Balch. 1993. Differential inhibition of multiple vesicular transport steps between the endoplasmic reticulum and trans Golgi network. *J. Biol. Chem.* 268:4216–4226.

Duden, R., G. Griffiths, R. Frank, P. Argos, and T.E. Kreis. 1991. β-COP, a 110 kDa protein associated with non-clathrin-coated vesicles and the Golgi complex shows homology to β-adaptin. *Cell.* 64:649–665.

Elrod-Erickson, M.J., and C.A. Kaiser. 1996. Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. *Mol. Biol. Cell.* 7:1043–1058.

Fiedler, K., and K. Simons. 1995. The role of N-glycans in the secretory pathway. *Cell.* 81:309–312.

Fiedler, K., M. Veit, M.A. Stammes, and J.E. Rothman. 1996. Bimodal interaction of coatomer with the p24 family of putative cargo receptors. *Science (Wash. DC).* 273:1396–1399.

Gaynor, E., and S.D. Emr. 1997. COPI-dependent anterograde transport: cargo-selective ER-to-Golgi protein transport in yeast COPI mutants. *J. Cell Biol.* 136:789–802.

Itin, C., R. Schindler, and H.-P. Hauri. 1995. Targeting of protein ERGIC-53 to the ER/ERGIC/*cis*-Golgi recycling pathway. *J. Cell Biol.* 131:57–67.

Itin, C., A.-C. Roche, M. Monsigny, and H.-P. Hauri. 1996. ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. *Mol. Biol. Cell.* 7:483–493.

Jackson, M.R., T. Nilsson, and P.A. Peterson. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* 121:317–333.

Kreis, T.E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:931–941.

Kuge, O., C. Dascher, L. Orci, T. Rowe, M. Amherdt, H. Plutner, M. Ravazzola, G. Tanigawa, J.E. Rothman, and W.E. Balch. 1994. Sar1 promotes vesicle budding from the endoplasmic reticulum but not Golgi compartments. *J. Cell Biol.* 125:51–65.

Lafay, F. 1974. Envelope viruses of vesicular stomatitis virus: effect of temperature-sensitive mutations in complementation groups III and V. *J. Virol.* 14: 1220–1228.

Lahtinen, U., U. Hellman, C. Wernstedt, J. Saraste, and R.F. Pettersson. 1996. Molecular cloning and expression of a 58-kDa cis Golgi and intermediate compartment protein. *J. Biol. Chem.* 271:4031–4037.

Letourneur, F., E.C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, S. Emr, H. Riezman, and P. Cosson. 1994. Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell.* 79:1199–1207.

Liener, I.E., N. Sharon, and I.J. Goldstein. 1986. The Lectins: Properties, Functions and Applications in Biology and Medicine. Academic Press, Inc./Harcourt Brace Jovanovich, publ. 71–73.

Lotti, L.V., M.R. Torrisi, M.C. Pascale, and S. Bonatti. 1992. Immunocytochemical analysis of the transfer of vesicular stomatitis virus G glycoprotein from the intermediate compartment to the Golgi complex. *J. Cell Biol.* 118:43–50.

Mizuno, M., and S.J. Singer. 1993. A soluble secretory protein is first concentrated in the endoplasmic reticulum before transfer to the Golgi apparatus. *Proc. Natl. Acad. Sci. USA.* 90:5732–5736.

Nilsson, T., M. Jackson, and P.A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell.* 58:707–718.

Oprins, A., R. Duden, T.E. Kreis, H.J. Geuze, and J.W. Slot. 1993. β-COP localizes mainly to the *cis*-Golgi side in exocrine pancreas. *J. Cell Biol.* 121:49–59.

Orci, L., D.J. Palmer, M. Amherdt, and J.E. Rothman. 1993. Coated vesicle assembly in the Golgi requires only coatomer and ARF proteins from the cytosol. *Nature (Lond.).* 364:732–734.

Ostermann, J., L. Orci, K. Tani, M. Amherdt, M. Ravazzola, Z. Elazar, and J.E. Rothman. 1993. Stepwise assembly of functionally active transport vesicles. *Cell.* 75:1015–1025.

Pelham, H.R.B. 1994. About turn for the COPs? *Cell.* 79:1125–1127.

Pind, S., C. Nuoffer, J.M. McCaffery, H. Plutner, H.W. Davidson, M.G. Farquhar, and W.E. Balch. 1994. Rab1 and Ca^{2+} are required for the fusion of carrier vesicles mediating endoplasmic reticulum to Golgi transport. *J. Cell Biol.* 125:239–252.

Plutner, H., A.D. Cox, S. Pind, R. Khosravi-Far, J.R. Bourne, R. Schwaninger, C.J. Der, and W.E. Balch. 1991. Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J. Cell Biol.* 115:31–43.

Plutner, H., H.W. Davidson, J. Saraste, and W.E. Balch. 1992. Morphological analysis of protein transport from the endoplasmic reticulum to Golgi membranes in digitonin permeabilized cells: role of the p58 containing compartment. *J. Cell Biol.* 119:1097–1116.

Rexach, M.F., and R.W. Schekman. 1991. Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114:219–229.

Rowe, T., M. Aridor, J.M. McCaffery, H. Plutner, and W.E. Balch. 1996. COPII vesicles derived from mammalian endoplasmic reticulum (ER) microsomes recruit COPI. *J. Cell Biol.* 135:895–911.

Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.

Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.

Saraste, J., G.E. Palade, and M.G. Farquhar. 1987. Antibodies to rat pancreas Golgi subfractions—identification of a 58-kD *cis* Golgi protein. *J. Cell Biol.* 105:2021–2029.

Saraste, J., U. Lahtinen, and B. Goud. 1995. Localization of the small GTP-binding protein Rab1 to early compartments of the secretory pathway. *J. Cell Sci.* 108:1541–1552.

Schimmoller, F., B. Singer-Kruger, S. Schroder, U. Kruger, C. Barlowe, and H. Riezman. 1995. The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1329–1339.

Schwaninger, R., C.J.M. Beckers, and W.E. Balch. 1991. Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266:13055–13063.

Schweizer, A., J.A.M. Fransen, T. Bachi, L. Ginsel, and H.-P. Hauri. 1988. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the *cis*-side of the Golgi apparatus. *J. Cell Biol.* 107:1643–1653.

Schweizer, A., J.A.M. Fransen, K. Matter, T.E. Kreis, L. Ginsel, and H. Hauri. 1990. Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. *Eur. J. Cell Biol.* 53:185–196.

Schweizer, A., K. Matter, C.A. Ketcham, and H. Hauri. 1991. The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and *cis*-Golgi. *J. Cell Biol.* 113:45–54.

Söhn, K., L. Orci, M. Ravazzola, M. Amherdt, M. Bremser, F. Lottspeich, K. Fiedler, J.B. Helms, and F.T. Wieland. 1996. A major transmembrane protein of Golgi-derived COPI coated vesicles involved in coatomer binding. *J. Cell Biol.* 135:12339–1248.

Stamnes, M.A., and J.E. Rothman. 1993. The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell.* 73:999–1005.

Stamnes, M.A., M.W. Craighead, M.H. Hoe, N. Lampen, S. Geromanos, P. Tempst, and J.E. Rothman. 1995. An integral membrane component of COPI-coated transport vesicles defines a new family of proteins involved in budding. *Proc. Natl. Acad. Sci. USA.* 92:811–815.

Tabas, I., and S. Kornfeld. 1979. Purification and characterization of rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem.* 254:11655–11663.

Tang, B.L., S.H. Low, H.-P. Hauri, and W. Hong. 1995. Segregation of ERGIC53 and the mammalian KDEL receptor upon exit from the 15°C compartment. *Eur. J. Cell Biol.* 68:397–410.

Tisdale, E.J., and W.E. Balch. 1996. Rab2 is essential for the maturation of pre-Golgi intermediates. *J. Biol. Chem.* 271:29372–29379.

Tisdale, E.J., J.R. Bourne, R. Khosravi-Far, C.J. Der, and W.E. Balch. 1992. GTP-binding mutants of Rab1 and Rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. *J. Cell Biol.* 119:749–761.

Waka, I., D. Rindress, P.H. Cameron, W.-J. Ou, J.J. Doherty II, D. Louvard, A.W. Bell, D. Dignard, D.Y. Thomas, and J.J.M. Bergeron. 1991. SSRα and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. *J. Biol. Chem.* 266:19599–19610.