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 (coatamer) (Letourneur et al., 1994; Aridor et al., 1995). Yeast mutant strains defective in the COPII 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) fusion protein that possesses the carboxyl-terminal KKXX motif binds COPI coat proteins. The binding of coatamer 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 facilitate binding to COPI, suggesting a bimodal interaction of proteins with coatamer (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 inhibit 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, 1995).

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.
presumed to be common (Ishii et al., 1995). It is unlikely that the block in protein transport represents a general pause in Golgi function, since the Golgi stack is still intact, in contrast to what is observed in some cells that undergo programmed cell death (Whitehead et al., 1996). What is also likely to be true is that the block in anterograde transport is a secondary effect of the block in protein export, and that COPI coats at the cis face of the Golgi complex are required to maintain this block in protein export at the trans face of the Golgi complex. The block in COPI coats removes COPII coats from the trans face of the Golgi complex, and this block in COPI coats allows the anterograde transport mechanism to correct the block in the packaging of p53/58 to the trans Golgi network.


the presence of antibody, we observe the accumulation of p58 in VTCs. The block in protein transport directly correlates with inhibition of COPII recruitment to the p53p58 cytoplasmic tail and VTCs in vitro. We propose that p58 and its homologue p53 are coatamer-binding proteins that participate in COPII-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 p53p58 (QGEEAAAKKFF) plus an NH2-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 50 mM Hepes, pH 7.2, 135 mM KOAc (25/125) and concentrated for use in the semintact 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′-GGCCGATCCATACAAATAGTGAAC- TACACAGG 3′. The resulting first-strand cDNA was amplified by PCR using the following primers, which generated 5′NdeI and 3′ BamHI sites: 5′-GGCATATGGGCGGGATCCGACAAAGGGGTCTCCGG 3′ and the reverse primer 5′-GGCGGTATTCTCAAAGAATTTTTTGCGC- GTTCG 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 solubel fraction was washed on a 12% SDS-PAGE and the band corresponding to the p53 protein, excised, and electroeluted.

Generation of GST–p53p58 Carboxyl Tail Fusion Protein

Oligonucleotides that corresponded to the cytoplasmic domain of p53p58 (forward primer introduced a BamHI site, 5′-GATCCTACAAAGAAGAAGCAGCTGCAAAA AAATTTTTCTG 3′, reverse primer introduced an EcoRI site, 5′-AATTCAGAAAAATTTTTTTCAGCTGCTTTGTTG 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 0.6OD600 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 refcentrified, 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 μM reduced glutathione. The resulting fusion protein was

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analyzed by SDS-PAGE and Western blot and was recognized by the anti-tail 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 Trans35S[35] for 10 min at the restrictive temperature (39.5°C) to accumulate VSV-G mutant in the ER. The cells were then perfused by wheeling and scraping, Eri 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 CaCl₂, 1 mM N-acetylglucosamine, an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate, and 0.2 U rabbit muscle creatine phosphokinase), and 5 μl rat liver cytosol and 5 μl of semintact cells (5 × 10⁶ 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 precubated 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 H (endo H) (Reid and Balch, 1992). The soluble fraction was then digested with 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 CaCl₂, 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 μg MTP/S (to promote constitutive ARF1 activation) were then added and the reactions shifted to 37°C 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 centrifuged at 16,000 g for 20 min at 4°C.
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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 A, 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 coatamer through either KKKXX-, KKKXXX-, 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.

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/p58 cytoplasmic tail (QQEAAKKFF) or a GST fusion protein containing the cytoplasmic tail of p53/p58 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 QOE 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 I). 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/p58 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/p58-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/p58. All subsequent studies use the affinity-purified antibody for analysis of the role of p53/p58 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
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 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.

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.
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 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 VTCs. Cells were then pelleted, held on ice in a Ca2+-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 Ca2+-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 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.

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 Man5 form, which appears in response to trimming by α-mannosidase I found in the cis region of Golgi stack (Beckers et al., 1987). The Man5 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 Man5 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 Ca2+-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 Ca2+ 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 Ca2+-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 Ca2+-dependent fusion of VTCs to the Golgi stack.
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, semintact 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 coordinates 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 di-phenylalanine 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 Rab11 (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 monoclonal 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,
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 coatamer recruitment by p53/p58, we analyzed COPI binding to the GST fusion protein containing the carboxyl terminus QQEEAACKFF 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 coatamer. 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 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).
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 coatamer, and incubated at 37°C in the presence of cytosol, ATP, and GTPyS, a nonhydrolyzable analog of GTP that constitutively activates the small GTPase ARF1 involved in coatamer binding to VTCs (Aridor et al., 1995). Previous studies have demonstrated that coatamer 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; Stammes and Rothman, 1993). In the absence of antibody, GTPyS...
Antitail antibody blocks binding of \( \beta \)-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, 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 \( \beta \)-COP in each fraction was determined by Western blotting and densitometry.

(B) Micromeres were prepared from whole cell homogenates as described in Materials and Methods, mixed with cytosol and 20 \( \mu \)M GTP\( \gamma \)S, 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 \( \beta \)-COP (arbitrary units) was determined by Western blotting and densitometry. In lane c, membranes were omitted from the cocktail.

**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 KXXX or KXXXX 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 Ca2+ (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 KXXX 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; Letourner 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 selective 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 polypeptide 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 KXXX, KKKXX, 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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