

# A Cytosolic Complex of p62 and rab6 Associates with TGN38/41 and Is Involved in Budding of Exocytic Vesicles from the *trans*-Golgi Network

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**Abstract.** TGN38/41, an integral membrane protein predominantly localized to the *trans*-Golgi network, has been shown to cycle to the plasma membrane and return to the TGN within 30 min. (Ladinsky, M. S., and K. E. Howell. 1992. *Eur. J. Cell Biol.* 59:92-105). In characterizing the proteins which associate with TGN38/41, a peripheral 62-kD protein, two forms of rab6 and two other small GTP-binding proteins were identified by coimmunoprecipitation. However, ~90% of the 62-kD protein is cytosolic and is associated with the same subset of small GTP-binding proteins. Both the membrane and cytoplasmic complexes were characterized by sizing column fractionation and velocity sedimentation. The membrane complex was ~250 kD (11.6 S) consisting of the cytosolic complex and a heterodimer of TGN38/41 (160 kD). The cyto-

solic complex was ~86 kD (6.1 S) consisting of p62 and one small GTP-binding protein. Preliminary evidence indicates that phosphorylation of the p62 molecule regulates the dissociation of the cytosolic complex from TGN38/41. Functionally the cytosolic p62 complex must bind to TGN38/41 for the budding of exocytic transport vesicles from the TGN as assayed in a cell-free system (Salamero, J., E. S. Sztul, and K. E. Howell. 1990. *Proc. Natl. Acad. Sci. USA.* 87:7717-7721). Interference with p62, rab6 or TGN38, and TGN41 cytoplasmic domains by immunodepletion or competing peptides completely inhibited the budding of exocytic transport vesicles. These results support an essential role for interaction of the cytosolic p62/rab6 complex with TGN38/41 in budding of exocytic vesicles from the TGN.

**T**HE molecular mechanism of vesicular transport, by which both proteins and lipids move from one subcellular compartment to another, has been a topic of intense investigation recently (for reviews see Rothman and Orci, 1992; Pryer et al., 1992; Mellman and Simons, 1992). Cell-free assays combined with genetic studies in *Saccharomyces cerevisiae* have allowed the identification of numerous molecules involved in the vesicular transport process. Many cell-free assays measure the entire process, departure of a marker from one compartment and arrival at the next (for reviews see Melançon et al., 1991; Sztul et al., 1992). However, vesicular transport can be divided into many individual processes and cell-free assays can be designed to study each individual step. Additional impetus to the vesicular transport field has come from the consistent finding that transport between each donor and acceptor compartment appears to be regulated by distinct members of the rab subfamily of the ras-related low molecular weight GTP-binding proteins (for reviews see Balch, 1990; Gruenberg and Clague, 1992; Pfeffer, 1992).

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We have been studying the budding of vesicles from an immobilized stacked Golgi fraction and have found that formation of exocytic transport vesicles from the TGN is both rapid and efficient (Salamero et al., 1990). To study the budding from the TGN in more detail, we have focused on a type 1, integral membrane protein of the TGN, TGN38; the cDNA was isolated, sequenced, and expressed by Luzio et al. (1990). Recently, an isoform of the TGN38 cDNA was identified, TGN41, which has an 8 base change, including a 5-bp insertion resulting in an extended cytoplasmic domain of 23 amino acids (Reaves et al., 1992). Most studies on TGN38 have used antibodies which recognize the luminal domain, as shown by epitope mapping of different polyclonal antibodies (Wilde et al., 1992). Since the luminal domain is identical in both isoforms, experiments to date have studied TGN38/41 (Luzio et al., 1990; Robinson, 1990; Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991; Ladinsky and Howell, 1992; Reaves and Bantings, 1992; Reaves et al., 1993; Humphrey et al., 1993; Bos et al., 1993).

Signals in the cytoplasmic domain of TGN38/41 have been shown to be responsible for its predominant TGN localization (Luzio et al., 1990; Humphrey et al., 1993; Bos et al., 1993). Although the predominant localization is the TGN, the molecule rapidly cycles to the PM and returns to its resident compartment within 30 min (Ladinsky and Howell,

1992; Reaves et al., 1993). These findings led us to investigate the proteins that associate with TGN38/41 to identify molecules involved in the trafficking process.

To amplify our signal, we started with a stacked Golgi fraction isolated from rat liver. The secretory proteins were removed by carbonate washing and the membranes were solubilized in CHAPS to form mixed detergent/lipid micelles. Using this preparation, we have identified a protein at 62 kD and a select subset of small GTP-binding proteins, including rab6 which coimmunoprecipitate with TGN38/41. The association of these proteins also has been demonstrated by two other methods, sizing column chromatography and velocity sedimentation in sucrose gradients. Rab6 has previously been localized to the Golgi complex and peripheral Golgi vesicles (Goud et al., 1990; Yang et al., 1992; Antony et al., 1992). Approximately 90% of p62 is cytosolic and is associated with four small GTP-binding proteins. The 10% of p62 which is bound to the Golgi membrane is in a complex with TGN38/41 and all of the GTP-binding proteins which are found associated with the cytosolic complex. Phosphorylation may regulate the dissociation of the p62 cytosolic complex from TGN38/41, and hence the membrane.

Since monomeric GTP-binding proteins are thought to function by catalyzing the assembly of interacting molecules (for review see Bourne et al., 1990; Sztul et al., 1992), the TGN38/41/GTP-binding protein complex is an excellent candidate for involvement in the vesicular transport process. Functional studies using a cell-free budding assay reveals that the p62 cytosolic complex must associate with the cytoplasmic domain of TGN38/41 for the formation of exocytic transport vesicles from the TGN.

## Materials and Methods

### Materials

Unless otherwise indicated, all chemicals were obtained from Sigma Chem. Co. (St. Louis, MO). CHAPS, alkaline phosphatase (from calf intestine), and secondary antibodies were purchased from Boehringer Mannheim (Indianapolis, IN). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). Peptides were synthesized by Macromolecular Resources (Fort Collins, CO).

### Antibodies

TGN38/41 antibodies were raised in a rabbit (#873) against a TGN38- $\beta$ -galactosidase fusion protein; the same antigen as used in Luzio et al. (1990). By epitope mapping, this antiserum recognized amino acids 69-74 present in the luminal domain of both TGN38 and 41 (antiserum 2, in Wilde et al. [1992]). Therefore, all immunoassays using this antibody detect both isoforms. p62 antibodies were raised against the band at 62 kD extracted from a 1D-gel of immunoprecipitates from a stacked Golgi fraction using antibodies against TGN38/41. Antibodies used in the cell-free assay were as described (Salamero et al., 1990). Antibodies against rab6 were the generous gift of B. Goud, Institut Pasteur, Paris, France.

### Pulse-Chase

NRK cells were preincubated in balanced salt solution containing vitamins for 45 min (GIBCO BRL, Gaithersburg, MD). They were then pulsed for 10 min with 100  $\mu$ Ci  $^{14}$ C-mixed amino acids (New England Nuclear, Boston, MA) per 100-mm<sup>2</sup> tissue culture dish in MEM containing 0.1 $\times$  the standard concentration of each of the labeled amino acids using a Select Amine Kit (GIBCO BRL). The pulse media was removed, the cells were washed twice in the preincubation media, and then incubated for various time points in MEM containing 10 $\times$  concentration of each of the amino acids which were labeled. After the chase, the cells were placed on ice and

solubilized in NET buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40). Immune serum (20  $\mu$ l) was added to each sample, incubated for 2 h at 4°C, and immune complexes were precipitated with 100  $\mu$ l of a 50% protein A-Sepharose slurry (Pharmacia LKB Biotechnology, Piscataway, NJ). Half of each immunoprecipitate was treated with neuraminidase; 100 mU of neuraminidase Type VIII in 100 mM Na-Acetate buffer, pH 5, 1 mM CaCl<sub>2</sub>, and 150 mM NaCl for 5 h at 37°C. The samples were solubilized, resolved by SDS-PAGE, enhanced with Entensify (New England Nuclear), dried, and exposed to KODAK XAR film (Eastman Kodak, Rochester, NY) for autoradiography.

### Fractionation

Stacked Golgi fraction was isolated from rat liver by the method of Lee-lavathi et al. (1970) (Salamero et al., 1990). All solutions contained a mix of proteolytic inhibitors: chymostatin, leupeptin, antipain, pepstatin 1  $\mu$ g/ml each. Cytosol was prepared from the 0.5 M sucrose fraction, which contains the soluble proteins. It was subjected to centrifugation (100 k g, 2 h) and the supernatant was concentrated to 40 mg/ml in an Amicon with a PM10 membrane. Both the stacked Golgi fraction and cytosol were aliquoted, frozen in liquid nitrogen, and stored at -80°C.

### 1D- and 2D-Gel Electrophoresis

SDS-PAGE (1D) was carried out using a 5-15% acrylamide gradient and the buffer system of Maizel (1971). Molecular weights were determined by use of SDS-PAGE standards (Biorad Labs., Richmond, CA). 2D-gel electrophoresis was according to Celis et al. (1990). In the first dimension, the isoelectric focusing gradient ranged from pH 3-10 and in the second dimension, the acrylamide gradient ranged from 10-15%. pI was measured using IEF standards (Biorad Labs).

### Immunoprecipitation

Stacked Golgi fraction (1 mg protein) was washed in 200 mM sodium carbonate, pH 11.0, for 30 min on ice and pelleted, 100 k g, 30 min. The pellet was resuspended in 2 ml CHAPS buffer (20 mM CHAPS, 20 mM HEPES-KOH, pH 6.8, 100 mM KCl, 0.3 M sucrose, containing the cocktail of proteolytic inhibitors), incubated on ice for 1 h, and then centrifuged (200 K g, 20 min) to remove insoluble material. The critical micellar concentration for CHAPS is 3-10 mM (Hjelmeland, 1980). Antiserum (20  $\mu$ l) was added to the supernatant and allowed to bind overnight at 4°C. Immune complexes were precipitated with sheep antibodies against the Fc domain of rabbit IgG which were covalently coupled to fibrous cellulose (Luzio, 1977). The immunoprecipitates were resuspended in 1.25% SDS, reduced with 1 mM DTT, boiled for 10 min, and alkylated with 5 mM iodoacetamide.

### Immunoblot and Ligand Blots

Nitrocellulose filters were blocked for 1 h in 10% defatted milk/PBS/0.02% sodium azide. The filters were then incubated overnight with primary antibody, washed, and incubated in secondary antibody for 2 h. The blots were visualized using either <sup>125</sup>I-protein A (ICN Biomedicals, Inc., Costa Mesa, CA) and autoradiography or anti-rabbit IgG coupled to alkaline phosphatase, followed by colorimetric detection reaction. GTP-ligand blotting was modified from Lapatina et al. (1987). Briefly, nitrocellulose filters were blocked for 1 h in 100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.3% Tween 20, and 4  $\mu$ M ATP (binding buffer) with 2% BSA. The filters were then incubated for 90 min in binding buffer with 0.5  $\mu$ Ci/ml [ $\alpha$ -<sup>32</sup>P]GTP (Amersham, Arlington Heights, IL), followed by extensive washing in the same buffer.

### Protease Protection

Stacked Golgi fraction was homogenized in an AA glass/Teflon homogenizer (Arthur H. Thomas, Philadelphia, PA) to form small vesicles. The vesicles (100  $\mu$ g protein) were treated with 3, 15, 30  $\mu$ g/ml trypsin for 30 min at 4°C in the presence or absence of 0.2% Triton X-100 in 100 mM HEPES, pH 6.8, 2 mM MgCl in a total volume of 50  $\mu$ l. Half of the sample was resolved by SDS-PAGE and the other half assayed for galactosyltransferase activity (Bretz and Stäubli, 1977) to assess the latency of the vesicles.

### Membrane Washing

Stacked Golgi fraction was pelleted through a 0.5 M sucrose cushion (150 K g, 30 min) and resuspended at 1 mg/ml in various wash conditions

for 1 h at 4°C. (1) 200 mM sodium carbonate, pH 11; (2) 1.0 M NaCl; (3) 6 M urea. Aliquots (100  $\mu$ l) were pelleted through a 0.5 M sucrose cushion (150 K g, 1 h) and the supernatants precipitated in ice cold 90% acetone. TX114 extractions were carried out as described (Bordier, 1981). All samples were resolved by SDS-PAGE.

### Gel Filtration

Gel-filtration chromatography was carried out using Sephacryl S100 (Pharmacia LKB Biotechnology). Samples of 50 mg protein of either carbonate washed stacked Golgi fraction solubilized in CHAPS buffer, or cytosol in CHAPS buffer were loaded onto the column and elution was with 20 mM Hepes-KOH, pH 6.8, 100 mM KCl, 0.3 M sucrose, 20 mM CHAPS containing the cocktail of proteolytic inhibitors. Fractions (3.0 ml) were collected, and aliquots were analyzed by ELISA. The column was calibrated with molecular weight standards: catalase, 232 kD; BSA, 66 kD; carbonic anhydrase, 29 kD; cytochrome c, 12 kD, and apoferritin, 6.5 kD.

### Velocity Sedimentation in Sucrose Gradients

Continuous 5–25% sucrose gradients were prepared in CHAPS buffer. Samples in CHAPS buffer plus 5% sucrose, (either 200  $\mu$ g cytosol or carbonate washed stacked Golgi fraction which was previously solubilized for 1 h at 4°C in CHAPS buffer and centrifuged to remove insoluble material) were layered onto the top of the gradient and centrifuged in an SW41 rotor (Beckman Instruments, Palo Alto, CA) at 40,000 rpm for 16 h. Fractions (330  $\mu$ l) were collected and analyzed either by ELISA or SDS-PAGE. Densities were determined using a refractometer (Bausch & Lomb Inc., Rochester, NY). S value standards were determined according to Siegel and Monty (1966) using bovine  $\alpha$ 2-macroglobulin, 20S; bovine liver catalase, 11.4 S; and BSA, 4.6 S as standards.

### ELISA

50- $\mu$ l aliquots from each of the sucrose gradient fractions were diluted with 50  $\mu$ l PBS and adsorbed to microtiter plates (Dynatech Labs, Inc., Chantilly, VA) overnight at 4°C. Plates were blocked and washed with 3% BSA/PBS/0.02% azide for 1 h. Primary antibodies were incubated for 4 h, followed by secondary antibodies conjugated with alkaline phosphatase for 2 h, developed, and read using a microtiter plate reader (Biorad Labs).

### In Vitro and In Vivo Phosphorylation

In vitro phosphorylation was carried out using a carbonate washed stacked Golgi fraction (100  $\mu$ g protein) which was pelleted through a 0.5 M sucrose cushion and resuspended in 100  $\mu$ l ddH<sub>2</sub>O and 25  $\mu$ l of 10 $\times$  reaction buffer (1 M NaCl, 0.5% TX-100, 500 mM Tris, pH 7.5, 20 mM MgCl<sub>2</sub>, 20 mM MnCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>) on ice for 10 min. Then 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Corp.) was added and incubated for 30 min at room temperature. The reaction was stopped by addition of 11.5  $\mu$ l 20% SDS, 752  $\mu$ l ice cold ddH<sub>2</sub>O, 50  $\mu$ l 1.0 M NaF. The sample was solubilized by addition of 100  $\mu$ l of 10 $\times$  20 mM CHAPS buffer and immunoprecipitated.

In vivo phosphorylation was carried out by incubating NRK cells with 5 mCi [<sup>32</sup>P]orthophosphate in phosphate free MEM (GIBCO BRL) for 2 h in the CO<sub>2</sub> incubator. The cells were washed 3 $\times$  in media, scraped, and pelleted. The pellet was solubilized in 20 mM CHAPS and immunoprecipitated. Half of each immunoprecipitate from both the in vitro and in vivo phosphorylations was treated with alkaline phosphatase for 1 h at 37°C.

### Cell-Free Budding Assay of Exocytic Transport Vesicles from the Trans-Golgi Network

The cell-free assay of budding from immobilized stacked Golgi fraction was carried out as described in Salamero et al. (1990). The magnetic core and shell beads used to immobilize the stacked Golgi fraction were the gift of John Ugelstad and Ruth Schmid, University of Trondheim, Norway. The exocytic transport vesicles of the budded fraction were previously characterized in Salamero et al. (1990). The amount of budding assayed by SDS-PAGE, transfer, immunoblotting, and detection with <sup>125</sup>I-protein A, was quantitated by use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In cell-free assays when antibodies were added to cytosol to test for a possible blocking effect, 50- $\mu$ l serum was added to 200  $\mu$ l cytosol and mixed at 4°C for 1 h, and then used in the cell-free assay. For immunodepletion of the cytosol, 50- $\mu$ l serum was added to 200  $\mu$ l cytosol and mixed at 4°C overnight. The immune-complexes were precipitated with 100  $\mu$ l cellu-

lose fibers which had affinity purified goat anti-rabbit IgG covalently bound. In cell-free assays when either antibodies were added or cytosolic volumes were changed by immunodepletion, the volume of the assay buffer was adjusted to maintain a constant volume.

For the reconstitution experiments, p62/rab complex was isolated from cytosol (10 mg protein in 300  $\mu$ l) on a Sephacryl S100 column (1.25 cm ID  $\times$  1 m). The column was eluted with PBS and aliquots of the fractions assayed for p62 by ELISA. The pooled peak fractions had a volume of 3.0 ml. In the cell-free assay, aliquots of the peak fraction corresponding to 0.5 $\times$  and the 1.0 $\times$  p62 concentrations were added with simultaneous reduction in assay buffer to maintain a constant assay volume.

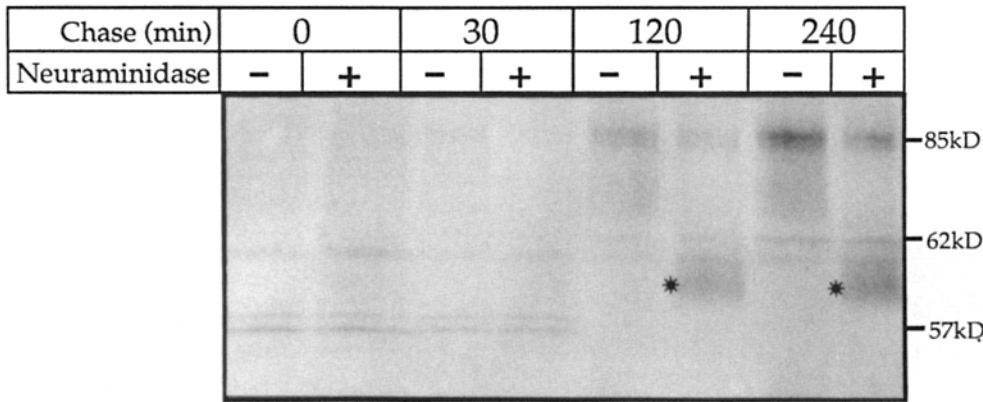
Peptides: (1) 12 carboxyl-terminal amino acids of TGN38 [CKASDYQRLNLK], (2) the 15 carboxyl-terminal amino acids of TGN41 [CKNLVLPADLFPNQEK], and (3) the 15-amino-terminal amino acids of TGN38 and TGN41 [LPSASKPNNTSSENNC] were added to the stacked Golgi fraction bound to the beads and to the cytosol at 100  $\mu$ M for 30 min at 4°C before combining the two fractions and initiation of the cell-free assay. All peptides were terminated with a cysteine which was used to couple them to keyhole limpet hemocyanin.

## Results

### Immunoprecipitation with TGN38/41 Antibodies Identify p62

In experiments to study the biogenesis of TGN38, a pulse-chase experiment was carried out in NRK cells. The cells were labeled for 10 min with <sup>14</sup>C-mixed amino acids (TGN38/41 have only one methionine and no cysteines in their mature sequences) and chased for various times in the presence of 10-fold excess unlabeled amino acids. Cells were solubilized with 1% NP-40 and immunoprecipitated; half of each immunoprecipitate was treated with neuraminidase. The analysis is shown in Fig. 1. At the completion of the pulse and through a 30-min chase, the immature forms of TGN38/41 are resolved as a doublet at  $\sim$ 57 kD. After a 120-min chase the immature forms are no longer detected and mature forms are resolved as a smeared doublet at  $\sim$ 85 kD (mature forms first detected at a 30-min chase). The immature forms are not neuraminidase-sensitive, while the mature forms are, and after neuraminidase digestion the mature forms shift to a broad band  $\sim$ 58–60 kD (indicated by asterisk). This demonstrates that TGN38/41 are polysialylated glycoproteins. A repeated observation of these experiments is that an additional band coimmunoprecipitates, seen in this experiment at 60 kD through 30 min and a second form at  $\sim$ 62 kD at later time points. Both the 60- and 62-kD forms are present through the 240-min chase. Neither form is neuraminidase-sensitive.

To characterize the 60/62-kD proteins, preparative immunoprecipitates from stacked Golgi fraction were carried out with antibodies against TGN38/41. A number of different detergent conditions were tested to solubilize the carbonate washed Golgi membranes and p62 remained associated with TGN38/41 using many solubilization protocols. We selected solubilization in 20 mM CHAPS because functional complexes of transmembrane proteins with cytoplasmic factors have been shown to remain intact in mixed CHAPS/lipid micelles (Rivnay et al., 1982; Rivnay and Metzger, 1982). The results of such an experiment are shown in Fig. 2A. An immunoprecipitate of TGN38/41 is resolved by SDS-PAGE and stained with Coomassie blue; a 62-kD protein is clearly visible along with the TGN38/41 doublet and the heavy chain of immunoglobulin. At steady state the 60–62 kD doublet is not seen; the 62-kD band predominates. The 62-kD band



**Figure 1.** Pulse-chase experiment with TGN38/41. NRK cells were pulsed for 10 min with  $^{14}\text{C}$ -mixed amino acids and chased for 0, 30, 120, and 240 min with 10-fold excess unlabeled amino acids. Cells were lysed and aliquots immunoprecipitated with antibodies against TGN38/41 and half of each immunoprecipitate was treated with neuraminidase. The immunoprecipitates were solubilized and resolved by SDS-PAGE. The gel was enhanced with Entensify, dried,

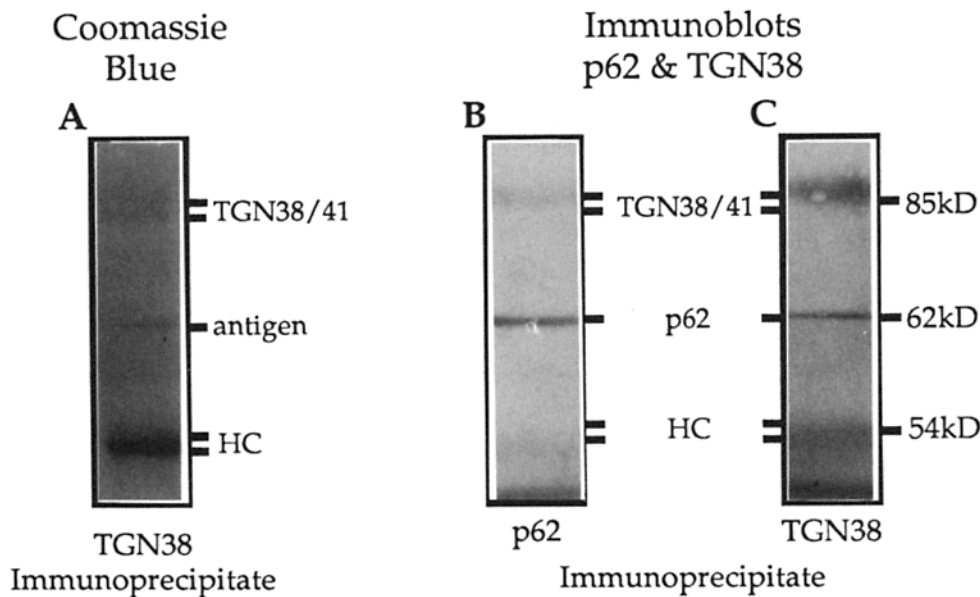
and autoradiography was carried out. Preimmune serum did not precipitate any bands (not shown). \* Denotes the neuraminidase product. TGN38/41 is difficult to label to a reasonable specific activity for autoradiography in a pulse-chase protocol, which indicates it has long half-life.

was excised from the gel and used for production of antibodies in a rabbit.

The p62 antiserum was characterized by immunoprecipitation from stacked Golgi fraction and compared directly with TGN38/41 immunoprecipitates. Both immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted sequentially using the two antibodies. Antibodies against p62 immunoprecipitated p62 and the TGN38/41 doublet (Fig. 2 *B*) while antibodies against TGN38/41 immunoprecipitated TGN38/41 and p62 (Fig. 2 *C*), as in the pulse-chase experiment. The antibodies against TGN38/41 do not recognize p62 on blots nor do the antibodies against p62 recognize TGN38/41 (see Fig. 4, *A* and *B*).

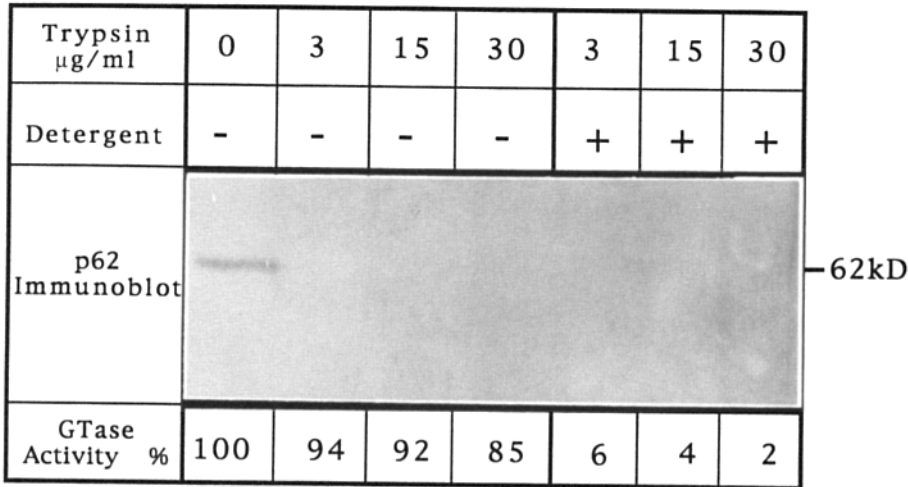
### ***p62 Is a Cytosolic Protein which Peripherally Associates with Golgi Membranes***

After the cell fractionation procedure used to isolate stacked Golgi fraction is carried out, ~90% of p62 is in the cytosolic fraction, ~10% in stacked Golgi fraction while only trace amounts are present in the heavier RER, mitochondrial and lysosomal fractions (data not shown). To confirm the cytosolic orientation of the p62 associated with Golgi membranes, protease sensitivity experiments were carried out in the presence and absence of detergent. Galactosyltransferase activity was used as the control to monitor the integrity of the vesicles derived from the stacked Golgi fraction (Fig. 3 *A*). In absence of detergent, p62 was completely degraded



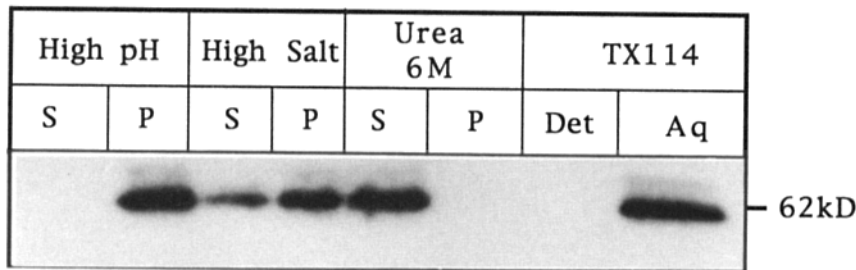
**Figure 2.** Identification of p62 and immunoprecipitations with p62 and TGN38/41 antisera. (*A*) Coomassie blue stained SDS-PAGE of a preparative immunoprecipitate of carbonate washed, CHAPS solubilized stacked Golgi fraction using antibodies against TGN38/41. The doublet at ~85 kD is TGN38/41. The band at ~62 kD which coimmunoprecipitated, labeled antigen, was excised and used for antibody production. HC is the heavy chain of the immunoglobulin used in the immunoprecipitation. (*B* and *C*) Immunoprecipitate from carbonate washed, CHAPS solubilized stacked Golgi fraction using antibodies against p62 (*B*) and TGN38/41 (*C*), resolved by SDS-PAGE, and transferred to nitrocellulose. Immunoblots were carried out consecutively with antibodies against TGN38/41 and p62.

A



**Figure 3.** Characterization of the association of p62 with Golgi membranes. (A) Stacked Golgi fraction was homogenized into vesicles and treated with 3, 10, or 30 μg/ml trypsin at 4°C in the absence or presence of 0.2% TX-100. The fractions were divided and half was resolved by SDS-PAGE followed by immunoblotting and the other half assayed for galactosyltransferase activity. The activity was 5,000 cpm transferred/min/mg and is expressed as % of nontreated fraction. (B) Stacked Golgi fraction was subjected to 200 mM sodium carbonate (*High pH*); 1 M NaCl (*High Salt*); 6 M Urea and then separated into supernatant (S) and pellet (P) fractions. TX114 extraction was carried out and then separated into detergent (Det) and aqueous (Aq) phases. The fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against p62.

B



by 3 μg/ml trypsin at 4°C for 30 min, conditions in which the galactosyltransferase activity was maintained. Only in the presence of detergents was the galactosyltransferase activity significantly diminished.

To study the association of p62 with the Golgi membrane, high pH and high salt washes were carried out as well as urea and TX-114 extractions (Fig. 3 B). p62 remained in the carbonate pellet, was minimally salt extracted, was recovered in the supernatant after 6 M urea, and distributed completely into the TX-114 aqueous phase. These data establish that p62 is a tightly associated peripheral protein oriented on the cytosolic face of the Golgi membrane.

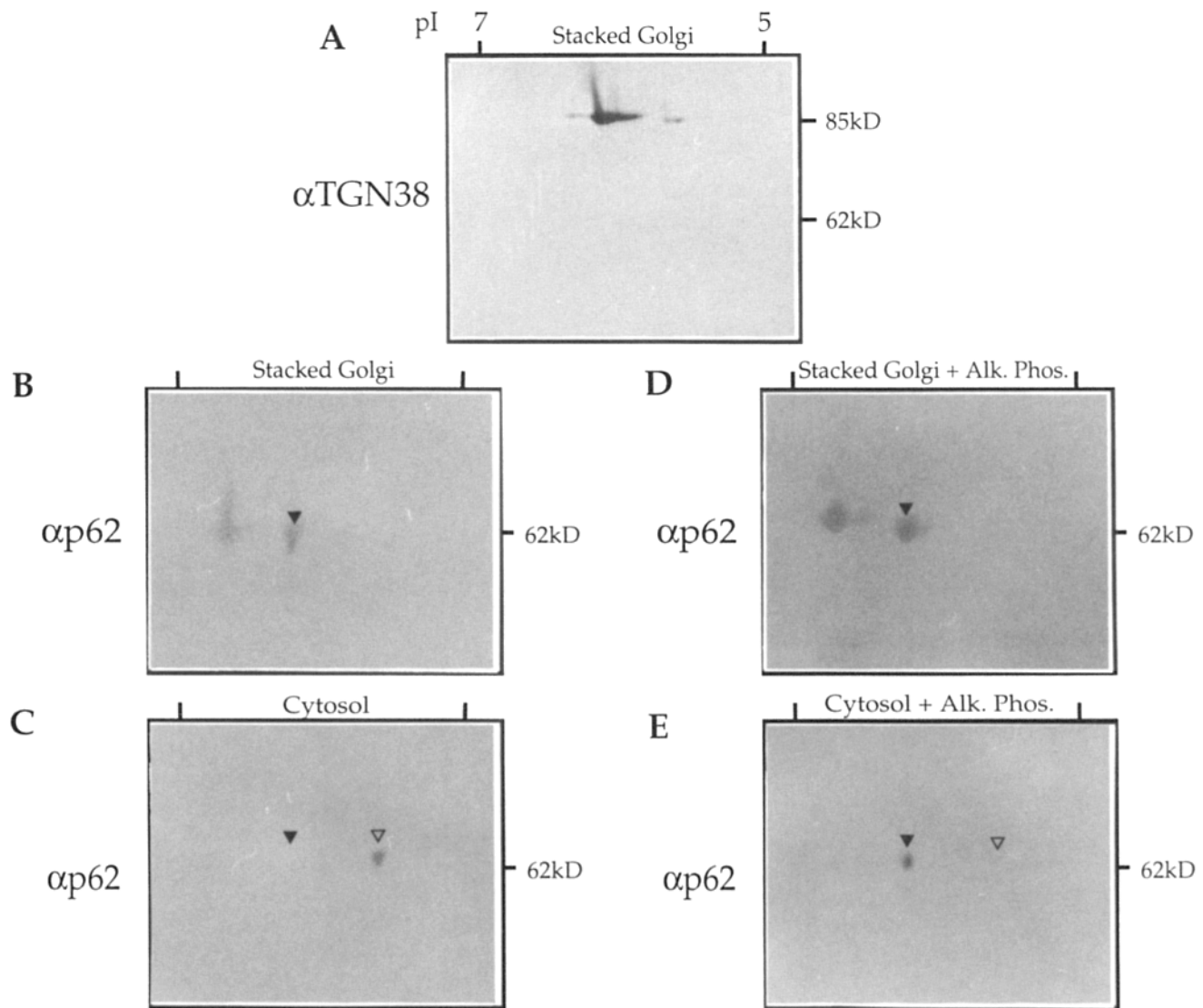
#### **Complexes Containing p62 in Both Golgi Membranes and Cytosol Include Small GTP-binding Proteins**

The complexes which contain p62 in both the Golgi membrane and in cytosol were characterized by: (a) 2D-gel analysis, (b) immunoprecipitation, (c) sizing column fractionation, and (d) velocity sedimentation in sucrose gradients. GTP-binding proteins also were characterized, since there are many indications that they are involved in the vesicular transport process.

**2D-Gel Analysis of Stacked Golgi Fraction and Cytosol.** To show the forms of TGN38/41 and p62, stacked Golgi

fraction and cytosol were resolved by 2D-gel electrophoresis, transferred, and probed with specific antibodies. An immunoblot with antibodies against TGN38/41 detect a heterogeneous protein in both charge (pI 6.4–6.1) and molecular weight (~85–88 kD) (Fig. 4 A). The charge heterogeneity is consistent with the polysialylation of TGN38/41 demonstrated in Fig. 1. An immunoblot of stacked Golgi fraction with antibodies against p62 detects two charge forms of p62 with pI's of 6.4 and 6.3 (Fig. 4 B). In contrast, in cytosol, only one charge form of p62 was detected (Fig. 4 C) and its pI, 6.1, is significantly more acidic than either form identified in stacked Golgi fraction (Fig. 4 B). More acidic forms often represent phosphorylation; to test this hypothesis an aliquot of each fraction was alkaline phosphatase treated. The forms of p62 associated with the Golgi membrane did not shift in pI (Fig. 4 D) while the form of p62 in cytosol shifted to a pI of 6.3—the same pI as the more acidic form associated with the Golgi membrane (Fig. 4 E). This data demonstrates that only the cytosolic form of p62 is phosphorylated. Note that antibodies against TGN38/41 do not recognize the p62 protein (Fig. 4 A) and antibodies against p62 do not recognize TGN38/41 (Fig. 4 B) on immunoblots; providing additional information that the two proteins are unrelated.

**Immunoprecipitation with Antibodies against Both TGN-**

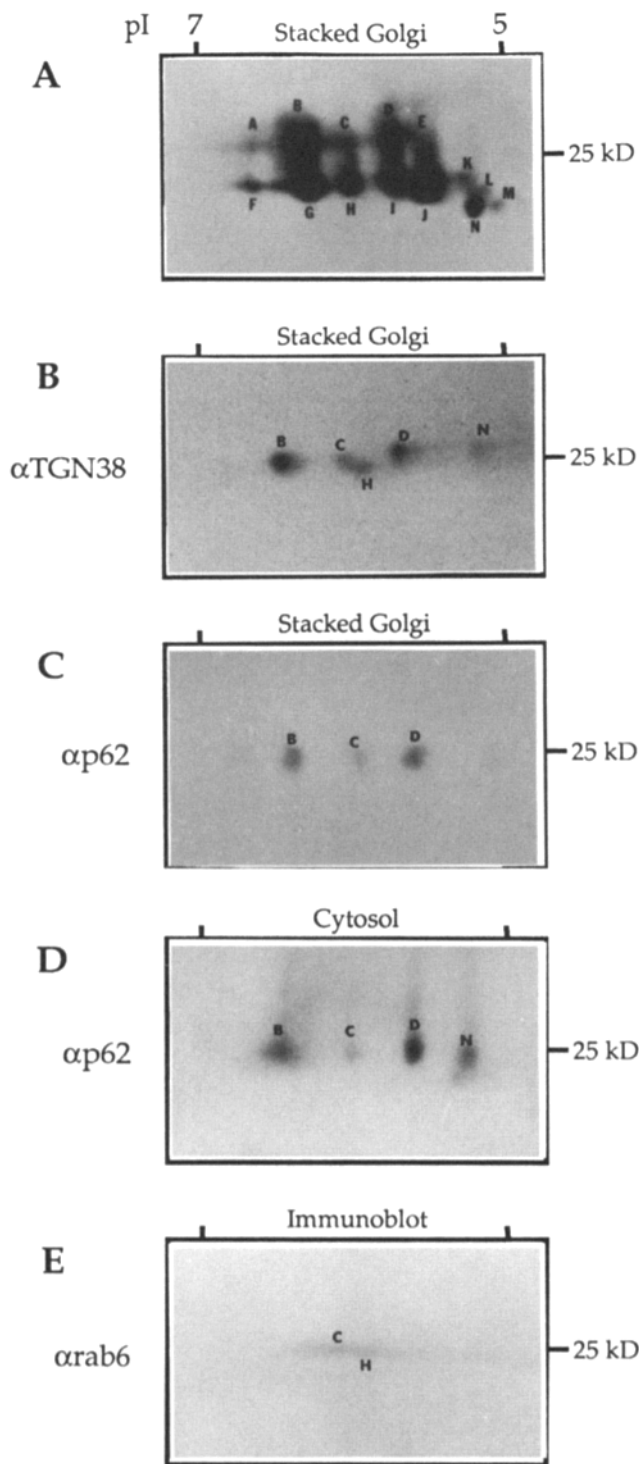


**Figure 4.** Characterization of TGN38/41 and p62 by 2D-gel analysis. Carbonate washed stacked Golgi fraction (*A*, *B*, and *D*) or cytosol (*C* and *E*) were resolved by 2D-gel electrophoresis, transferred to nitrocellulose, and immunoblotted. (*A*) Stacked Golgi with antibodies against TGN38/41; (*B*) stacked Golgi with antibodies against p62; (*C*) cytosol with antibodies against p62; (*D*) alkaline phosphatase-treated stacked Golgi with antibodies against p62; and (*E*) alkaline phosphatase-treated cytosol with antibodies against p62. Filled arrow head represents pI of 6.3 and open arrow head represents pI of 6.1.

**38/41 and p62 Coprecipitate a Discrete Set of Small GTP-binding Proteins.** Immunoprecipitates from carbonate washed, CHAPS solubilized stacked Golgi fraction and from cytosol with TGN38/41 and p62 antisera were resolved by 2D-gel electrophoresis, transferred to nitrocellulose and probed with [ $\alpha^{32}$ P]GTP to assay for coprecipitation of GTP-binding proteins. For comparison, the small GTP-binding proteins associated with the carbonate washed stacked Golgi fraction are shown in Fig. 5 *A*. Surprisingly, at least 14 GTP-binding proteins with molecular weights of  $\sim 25$  kD are resolved, labeled A–N (note that ARF's are in the 21-kD range and are not shown in this panel). The ligand blot of an immunoprecipitate with antibodies against TGN38/41, reveal five small GTP-binding proteins "B," "C," "D," "H," and "N," present in different proportions (Fig. 5 *B*). This is a simplified pattern compared with that of the en-

tire Golgi fraction. An immunoprecipitate with antibodies against p62, coprecipitate three of the same small GTP-binding proteins as antibodies against TGN38/41 ("B," "C," and "D") (Fig. 5 *C*). Remarkably, p62 immunoprecipitates from cytosol coprecipitate four of the same small GTP-binding proteins as TGN38/41 ("B," "C," "D," and "N") (Fig. 5 *D*). "H" and "N" vary most in concentration. "N" is much higher in the cytosolic complex and minimally present in the membrane complexes. "H" is discussed below.

Since rab6 had already been localized to the Golgi complex and its associated vesicles, it was logical to determine if rab6 was one of the major GTP-binding proteins associated with TGN38/41 and p62. Therefore, immunoblots were carried out with antibodies against rab6 on the filter in *B* and the two slightly skewed spots of "C" and "H" were both positive. This indicates that some processing of rab6 has



**Figure 5.** Characterization of the small GTP-binding proteins associated with the stacked Golgi-fraction, TGN38/41, and p62. Carbonate washed, CHAPS solubilized, stacked Golgi fraction and cytosol were immunoprecipitated with specific antisera. The carbonate washed stacked Golgi fraction and the immunoprecipitates were resolved by 2D-gel electrophoresis, transferred to nitrocellulose and ligand blotted with [ $\alpha^{32}$ P]GTP or immunoblotted. (A) Carbonate washed stacked Golgi fraction; (B) stacked Golgi fraction immunoprecipitated with antibodies against TGN38/41; (C) stacked Golgi fraction immunoprecipitated with antibodies against p62; (D) cytosol fraction immunoprecipitated with antibodies against p62; and (E) immunoblot of filter in B with antibodies

taken place when it is bound to TGN38/41. The other GTP-binding spots "B," "D," and "N" are not identified (see Discussion).

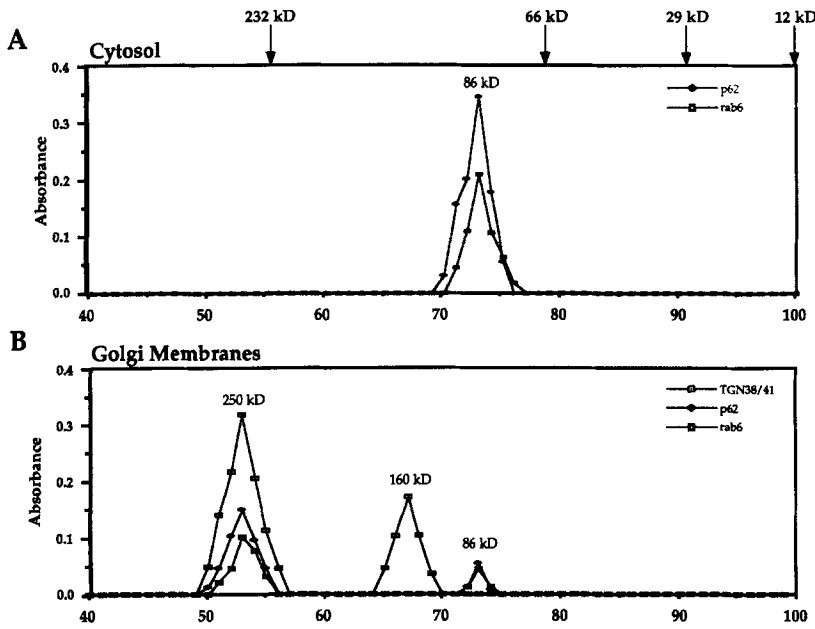
Taken together with the data in Fig. 2, these data indicate that the complex associated with the Golgi membranes consists of TGN38/41, p62, two forms rab6, and at least two other small GTP-binding proteins, either singly or in combinations. The observation that p62 forms a complex in cytosol with the same limited subset of small GTP-binding proteins, with rab6 present in only one form, suggests a dynamic interaction between the p62 cytosolic complex and TGN38/41 in the Golgi membrane.

**Gel-Filtration Chromatography of Complexes in Cytosol and Golgi Membrane.** The approximate sizes of the p62 cytosolic and membrane complexes were determined by chromatography using a Sephacryl S100 column. Either cytosol or the carbonate washed, CHAPS solubilized stacked Golgi fraction was loaded onto the column, eluted in CHAPS buffer and fractions collected. Aliquots of the fractions were analyzed for p62, rab6, and TGN38/41 distribution by ELISA. In cytosol, p62 and rab6 coelute at a molecular weight of  $\sim 86$  kD (Fig. 6 A), indicating that only one GTP-binding protein associates with a molecule of p62 at any one time. From the solubilized stacked Golgi fraction, p62, rab6, and TGN38/41 coelute as a large complex of  $\sim 250$  kD (Fig. 6 B). Not all of TGN38/41 is in this complex,  $\sim 30\%$  elutes at  $\sim 160$  kD with neither p62 nor rab6. In addition, a small proportion of p62 and rab6 coelute at 86 kD, in the same position as the cytosolic complex, suggesting they have dissociated during the fractionation. The 160-kD peak was pooled, taken to 2% SDS, boiled for 10 min, and rechromatographed. All of the TGN38/41 molecules are now eluted at a molecular weight of  $\sim 80$  kD, indicating that the total population of TGN38/41 molecules are dimeric (data not shown). The simplest stoichiometry for the membrane complex, therefore, would be one copy of the TGN38/41 dimer (160 kD) and one copy of the p62, small GTP-binding protein complex (86 kD).

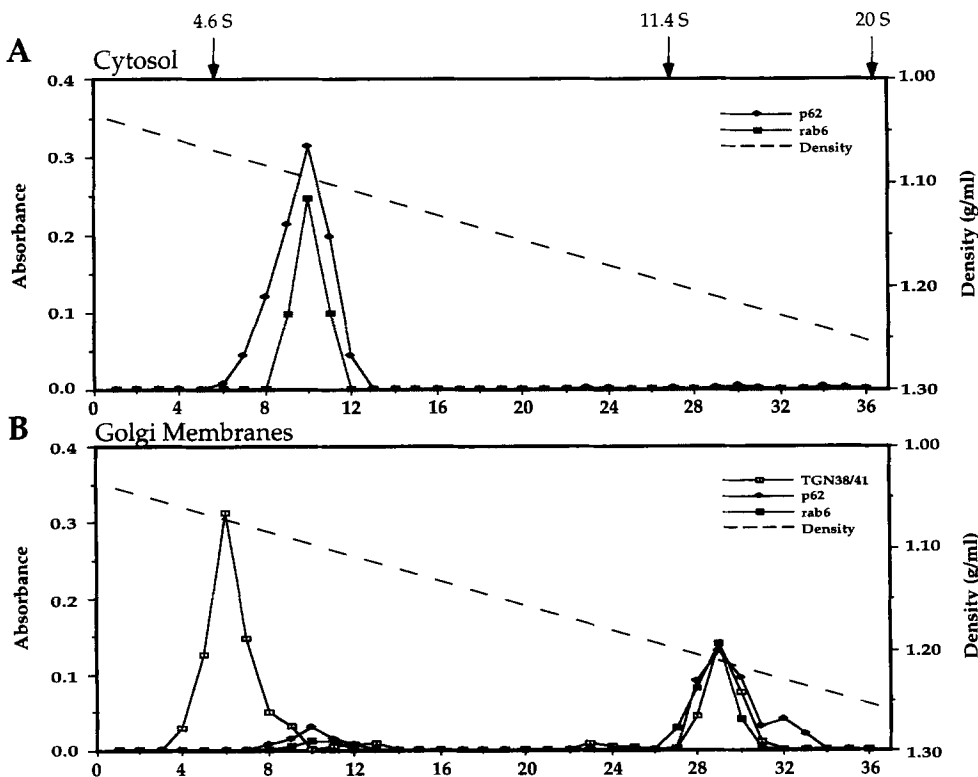
**Velocity Sedimentation of Complexes in Cytosol and Golgi Membrane.** The cytosolic and membrane complexes of p62 were further characterized by velocity sedimentation in sucrose gradients in the presence of CHAPS. Fractions from the gradients were analyzed for p62, rab6, and TGN38/41 distribution by ELISA. The cytosol complex of p62 and rab6 has a sedimentation coefficient of  $\sim 6.1$  S, (Fig. 7 A) which corresponds to a globular protein of  $\sim 86$  kD. In the membrane p62, rab6, and TGN38/41 cosediment as complex with a sedimentation coefficient of  $\sim 11.6$  S (Fig. 7 B), which corresponds to a globular protein of  $\sim 232$  kD. As with the column fractionation, not all of TGN38/41 sediments with p62 and rab6, but in the sucrose gradient a larger proportion of TGN38/41 is unassociated with p62 and rab6 and has a sedimentation coefficient of  $\sim 4.7$  S. Also, as in the column fractionation, a small proportion of p62 and rab6 has dis-

against rab6. In controls, GTP-binding proteins were not coimmunoprecipitated (data not shown). Controls for the CHAPS solubilized stacked Golgi fraction were immunoprecipitated using preimmune serum to TGN38/41 and p62 as well as antibodies against the pIgA-R. Controls for the cytosolic fraction were immunoprecipitated using antibodies against tubulin.





**Figure 6.** Sizing of the CHAPS solubilized cytosolic and membrane p62 complexes using a Sephacryl S100 column. Samples of 200  $\mu$ g protein of either cytosol in CHAPS buffer (A) or carbonate washed, CHAPS solubilized stacked Golgi fraction (B) were loaded onto a Sephacryl S100 column and eluted with 20 mM Hepes-KOH, pH 6.8, 100 mM KCl, 0.3 M sucrose, 20 mM CHAPS containing the cocktail of proteolytic inhibitors. Fractions (3.0 ml) were collected and aliquots analyzed by ELISA using antibodies against p62, rab6, and TGN38/41. (A) ELISA results of p62(—◆—) and rab6(—■—) on the column fractions. (B) ELISA results of p62(—◆—) and rab6(—■—) and TGN38/41 (—□—) on the column fractions. The elution profile of molecular weight standards is shown at the top of A. The distribution of the antigens was confirmed by SDS-PAGE of aliquots of the fractions, transfer to nitrocellulose and sequential ligand blotting with [ $\alpha^{32}$ P]GTP and immunoblotting with antibodies against p62. The distribution of the total GTP-binding proteins was much wider than the peak of p62 and rab6 (data not shown).



**Figure 7.** Distribution of the cytosolic and membrane p62 complexes in velocity sedimentation gradients. Continuous 5–25% sucrose gradients were prepared in CHAPS buffer. Samples were prepared in CHAPS buffer containing 5% sucrose, layered onto the top of the gradients, and centrifuged at 40,000 rpm for 16 h. Fractions (330  $\mu$ l) were collected and aliquots analyzed by ELISA using antibodies against p62, rab6, and TGN38/41. (A) ELISA results of p62(—◆—) and rab6(—■—) on the gradient fractions and density (— - —). (B) ELISA results of p62(—◆—), rab6(—■—), TGN38/41(—□—) on the gradient fractions, and density (— - —). The distribution of S value standards in the gradient is shown at the top of A. The distribution of the antigens was confirmed by SDS-PAGE of aliquots of the fractions, transfer to nitrocellulose and sequential ligand blotting with [ $\alpha^{32}$ P]GTP and immunoblotting with antibodies against p62. The distribution of the total GTP-binding proteins was much wider than the peak of p62 and rab6 (data not shown).



sociated and has a sedimentation coefficient of  $\sim 6.1$  S, the same value as the cytosolic complex and larger than the free TGN38/41.

In summary, results from coimmunoprecipitation, sizing column fractionation, and velocity sedimentation in sucrose gradients are in accordance. The complex in cytosol is composed of p62 and rab6 (or one of three other GTP-binding proteins) and is  $\sim 86$  kD. The complex in the Golgi membrane includes the cytosolic complex plus TGN38/41 and is  $\sim 250$  kD. However, one of the GTP-binding proteins present in the cytosolic complex ("N") is minimally present in the membrane complex. Notably, two forms of rab6 are present in the membrane complex while only one form is associated with the cytosolic complex.

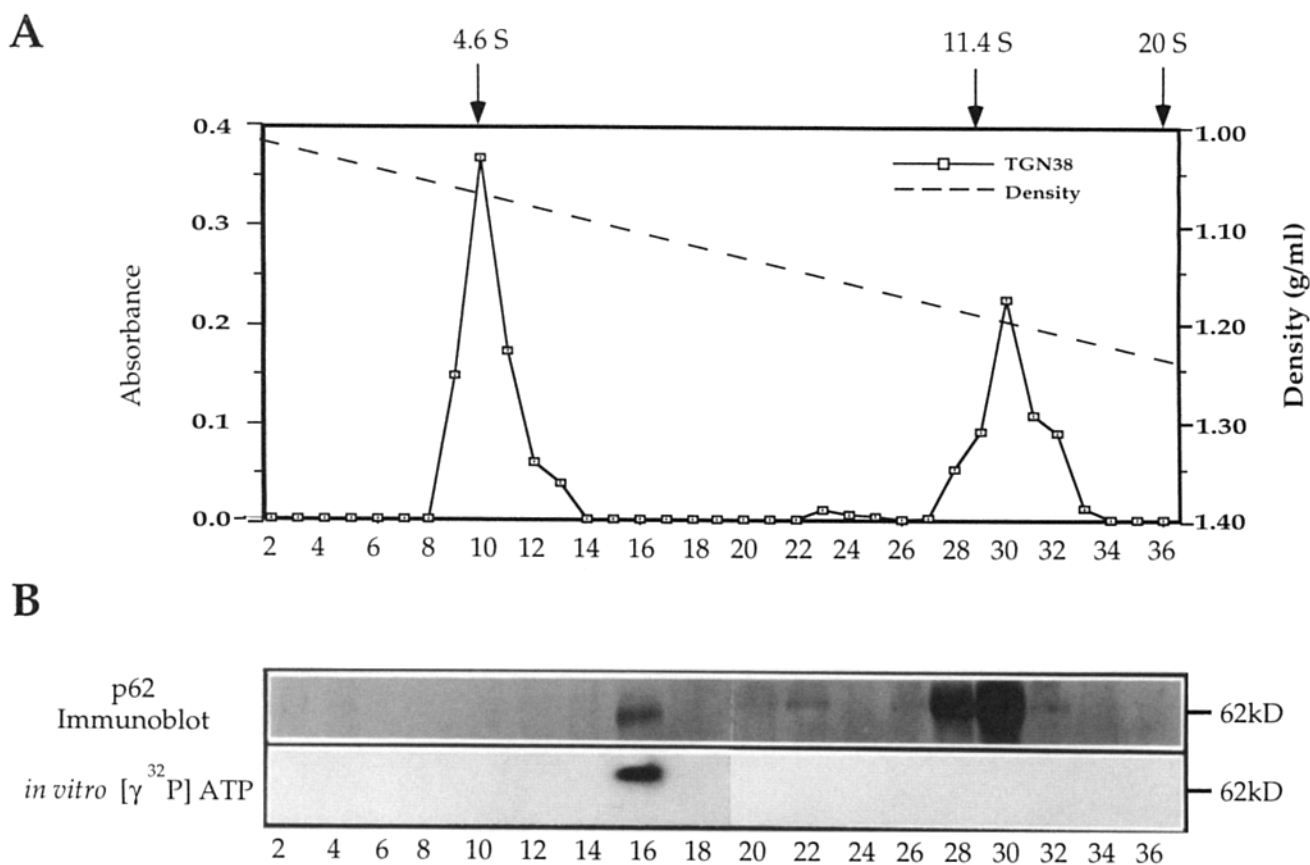
### Phosphorylation of p62

Since by 2D analysis p62 is found as three charge forms with the most acidic being in the cytosol and that only the cytosolic form is sensitive to alkaline phosphatase, we hypothesized that phosphorylation might regulate the membrane association of the cytosolic complex.

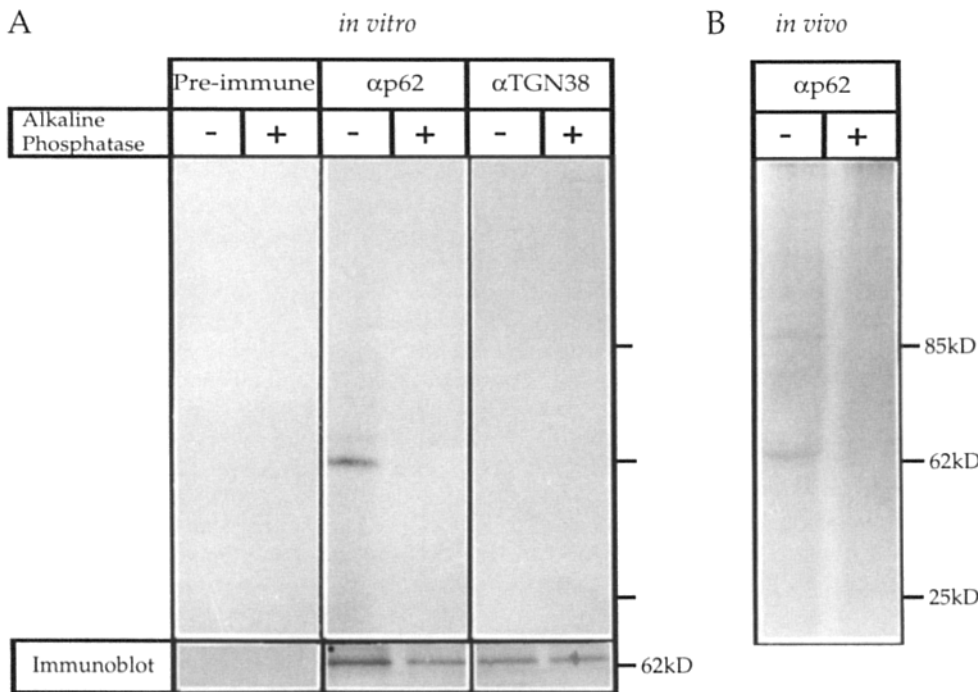
To directly test if the membrane form of p62 can be phosphorylated, stacked Golgi fraction was incubated with

$[\gamma^{32}\text{P}]\text{ATP}$  without addition of cytosol or exogenous kinases for 30 min at room temperature. The fraction was then CHAPS solubilized and loaded onto sucrose gradients. After centrifugation, the gradients were fractionated. Most of each fraction was immunoprecipitated with antibodies against p62 and the remainder was used for ELISA to follow TGN38/41. The immunoprecipitates were resolved by SDS-PAGE, transferred, and immunoblotted with antibodies against p62. The majority of p62 sedimented at 11.6 S (Fig. 8, A and B), i.e., the same velocity as the TGN38/41, p62, rab6 complex characterized in Fig. 7 B. However, a small amount of p62 sedimented at 6.1 S, i.e., the velocity of the cytosolic complex. Autoradiography of the nitrocellulose filter showed that only the p62 with the sedimentation velocity of the cytosolic complex was phosphorylated. Consistent with the data in Fig. 7, the more rapidly sedimenting complex containing the unlabeled p62 also contained TGN38/41 ( $\sim 30\%$ ) (Fig. 8 A). This data suggests that the phosphorylation of p62 displaced the p62 complex from TGN38/41 to the cytosolic form.

If this is the case, then the phosphorylated form of p62 should not associate with TGN38/41, and should not be immunoprecipitated with antibodies against TGN38/41. After *in vitro* phosphorylation, stacked Golgi fraction was



**Figure 8.** Identification of a phosphorylated form of p62 on sucrose density gradients. Carbonate washed stacked Golgi fraction was phosphorylated *in vitro*, solubilized in CHAPS and fractionated by velocity sedimentation on a continuous 5–20% sucrose gradient. Fractions (330  $\mu\text{l}$ ) were collected, 250  $\mu\text{l}$  of each sample was immunoprecipitated with antibodies against p62 (B), and the remainder used to assay TGN38/41 by ELISA (A). (A) Distribution of TGN38/41 (—□—) and density profile (----). The distribution of S value standards in the gradient is shown at the top of A. (B) The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against p62 (upper panel) followed by autoradiography (lower panel) of the same nitrocellulose filter.



**Figure 9.** The phosphorylated form of p62 does not interact with TGN38/41. (A) Carbonate washed stacked Golgi fraction was phosphorylated in vitro, solubilized, and immunoprecipitated with either preimmune, p62, or TGN38/41 antisera. Half of each immunoprecipitate was treated with alkaline phosphatase. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against p62 (lower panel), followed by autoradiography of the same nitrocellulose filter (upper panel). \* Denotes the phosphorylated form of p62 which is alkaline phosphatase-sensitive. i.e., Note that a small amount of the p62 appears to be hyperphosphorylated. (B) NRK cells were labeled in vivo with [<sup>32</sup>P]orthophosphate, washed, solubilized, and immunoprecipitated with p62 antisera. Half of the immunoprecipitate was alkaline phosphatase treated. Samples were resolved by SDS-PAGE followed by autoradiography. p62 is labeled and is alkaline sensitive. A second band just above 85 kD is also seen, which is probably the p62-small GTP-binding protein complex which did not dissociate.

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CHAPS solubilized, immunoprecipitated with preimmune p62 serum and antibodies against p62 and TGN38/41; half of each immunoprecipitate was treated with alkaline phosphatase. The immunoprecipitates were resolved by SDS-PAGE, transferred, and both immunoblotting and autoradiography carried out on the same filter (Fig. 9 A). Both antibodies immunoprecipitated approximately the same amount of p62, as shown on the immunoblot. However, the autoradiograph shows that only p62 antibodies can immunoprecipitate a labeled form of p62 and this form is alkaline phosphatase-sensitive. The labeled form also is apparent on the immunoblot of the p62 immunoprecipitate as a light band of slightly higher molecular weight (indicated by asterisk) which is also alkaline phosphatase-sensitive. These studies indicate that when p62 is phosphorylated, it is no longer associated with TGN38/41 and hence with the Golgi membrane. It is important to note that neither TGN38/41 nor the small GTP-binding proteins were phosphorylated in this assay.

To assure that p62 is phosphorylated in vivo, NRK cells were incubated with [<sup>32</sup>P]orthophosphate, washed, solubilized, and immunoprecipitation with antibodies against p62 carried out. Fig. 9 B shows that p62 is phosphorylated and alkaline phosphatase-sensitive, although the level of phosphorylation is not extensive. Taken together with the 2D-gel analysis showing only the cytosolic form is alkaline phosphatase-sensitive (Fig. 4, B-E), we conclude that p62 is an in vivo substrate for phosphorylation.

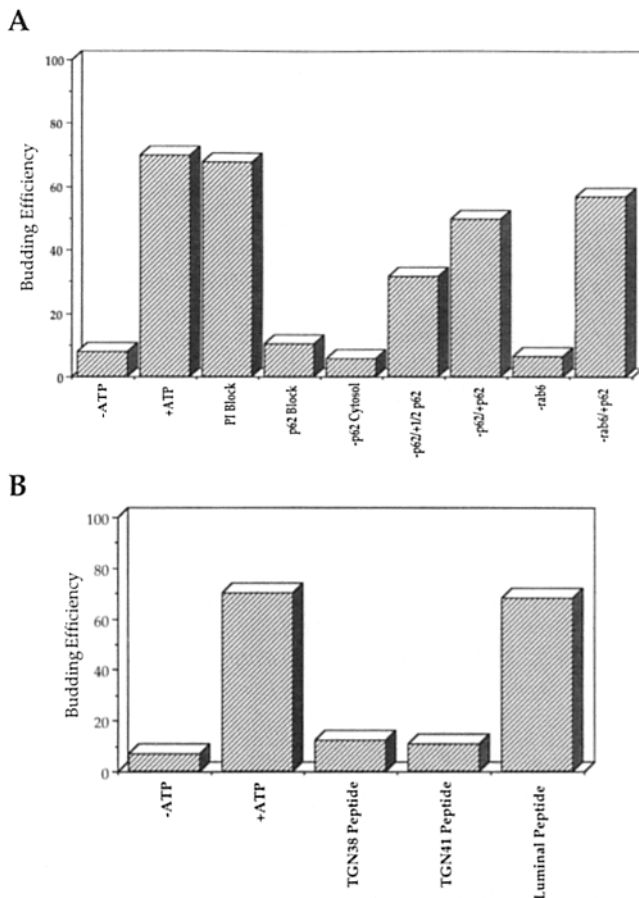
#### Cell-Free Assay to Test Function of the Cytosolic p62 Complex

A number of findings led us to test the role of the cytosolic complex in the vesicular transport process. First, TGN38/41 rapidly moves to the plasma membrane and back (Ladinsky

and Howell, 1992; Reaves et al., 1993). Second, TGN38/41 forms a complex with p62 and small GTP-binding proteins, molecules which have been shown to be involved in regulation of vesicular transport. Third, the cytosolic complex appears to reversibly associate with TGN38/41 via phosphorylation of the p62 molecule. We used the cell-free assay which reproduces exocytic vesicle budding from the TGN (Salamero et al., 1990) to test for a function of the cytosolic complex.

In this assay a stacked Golgi fraction is immobilized on magnetic beads and in the presence of cytosol, an ATP-regenerating system and 37°C, vesicles destined to different subcellular localizations are formed. The Golgi fraction remaining on the beads is retrieved and the budded vesicles remain in the supernatant. Budding from the TGN is both rapid and efficient; within 10 min 70% of mature-sialylated proteins destined to the plasma membrane bud into a distinct vesicle population. Salamero et al. (1990) characterized two proteins which bud from the TGN and are present in vesicles destined to the plasma membrane: the polymeric IgA-receptor (pIgA-R)<sup>1</sup>, a type 1 transmembrane protein, and transferrin, a soluble luminal protein. In the following experiments the budding of exocytic transport vesicles was quantitated by assay of the amount of mature pIgA-R in the budded fraction (Fig. 10, A and B). Budding in the presence of the complete cell-free system was ~70% efficient. When the ATP-regenerating system and cytosol were omitted, the background budding was ~10%. Addition of preimmune sera (to p62) to the cytosol before the addition of the ATP-regenerating system did not reduce the budding efficiency. In contrast, addition of antisera against p62 reduced the budding to background levels.

1. Abbreviations used in this paper: GDI, GDP dissociation inhibitor; pIgA-R, polymeric IgA-receptor.



**Figure 10.** Functional analysis of the cytosolic p62 complex using cell-free budding assay. Budding of vesicles from immobilized stacked Golgi fraction was carried out as described in Salamero et al. (1990). The budded fraction was resolved SDS-PAGE, transferred to nitrocellulose, immunoblotted with antibodies against the exoplasmic domain of the pIgA-receptor, detected with  $^{125}\text{I}$ -protein A, and quantitated using a PhosphorImager. (A) Preimmune-sera or antisera were added to the cytosol for 1 h on ice before addition of the cytosol to the cell-free assay. Immunodepletion was carried out by addition of p62 or rab6 antiserum to cytosol overnight at 4°C and immunocomplexes removed with anti-rabbit IgG cellulose. The cytosol after immunodepletion was assessed by SDS-PAGE, transfer, immunoblotting, and shown to be depleted of p62 and rab6, and the immunoprecipitates were shown to contain p62 and small GTP-binding proteins (data not shown). (B) Peptides were added to the stacked Golgi fraction and cytosol at 100  $\mu\text{M}$  for 30 min at 4°C before the fractions were mixed and the cell-free assay initiated. (*TGN38*) Peptide of the 12 carboxy-terminal amino acids of TGN38; this sequence is shared by TGN41, but is not carboxy-terminal in TGN41. (*TGN41*) Peptide of the 15 carboxy-terminal amino acids of TGN41; this sequence is unique to TGN41. (*Luminal Peptide*) Peptide of the 15 amino-terminal amino acids both TGN38 and TGN41.

To confirm the importance of the cytosolic complex in the budding process, immunodepletion and reconstitution of the cytosol was carried out. Cytosol was immunodepleted with either antibodies against p62 or rab6 before addition to the cell-free assay. Immunodepletion of cytosol with both antibodies resulted in inhibition of budding to background levels. Progressive restoration of the budding reaction was accomplished by the addition of increasing amounts of the

fraction containing the p62 complex from the Sephacryl S100 column (fractions 71–75, Fig. 6). At 0.5 $\times$ , the cytosolic equivalent budding was restored to 45% of control while the 1.0 $\times$ , the cytosolic equivalent, restored budding to 75% of control. Taken together this data indicates that the p62/rab6 complex in cytosol is necessary for the formation of exocytic transport vesicles from the TGN. Addition of antibodies to p62 is enough to disrupt the required interactions and inhibit vesicle formation.

The interaction between the p62/rab6 cytosolic complex with the Golgi membrane must be via the cytosolic domains of TGN38/41. Therefore, peptides of these cytosolic domains should compete for this interaction and also inhibit the budding reaction. To test this, peptides of the cytoplasmic domain of both TGN38 and TGN41 as well as a peptide against the NH<sub>2</sub>-terminal (luminal domain) of TGN38/41 were added to both the stacked Golgi fraction bound to the beads and the cytosol for 30 min at 4°C before mixing the two fractions and initiation of the cell-free assay (Fig. 10 B). Each cytosolic domain peptide individually reduced the budding to the control level, while the luminal domain peptide had no effect. This data indicates that each functional complex involves both TGN38 and TGN41 and indicates that the TGN38/41 dimer (Fig. 6) is a heterodimer. If homodimers were involved one would expect the TGN38 peptide to inhibit ~50% and the TGN41 peptide to inhibit ~50% and only a mix of the peptides could reduce the budding to the control level. The peptide competition data provides further evidence for the dynamic interaction between the p62/rab6 cytosolic complex and TGN38/41 in the budding of exocytic transport vesicles.

## Discussion

A cytosolic complex of p62 and rab6 (or one of two other small GTP-binding proteins) associates with an integral membrane protein of the TGN, TGN38/41. This association is essential for the budding of exocytic transport vesicles from the TGN assayed in a cell-free system.

Our proposed model of this process is shown in Fig. 11. At any one time point, ~90% of p62 is tightly associated with rab6 (or one of three other small GTP-binding proteins) in the cytosol and 10% is interacting with TGN38/41, a heterodimeric transmembrane protein of the TGN. The membrane-bound complex also contains rab6 (or one of the other two GTP-binding proteins that are associated with p62 in the cytoplasm), but some of the rab6 molecules have been modified. p62 is phosphorylated both in vivo and in vitro. In vitro data indicates that phosphorylation of p62 regulates the dissociation of the complex from TGN38/41 to the cytosol. While in the cytosol p62 remains phosphorylated, since by 2D-gel analysis the total pool is a single acidic form which is sensitive to alkaline phosphatase digestion. One would predict that association of the complex with TGN38/41 requires dephosphorylation of p62 because the membrane-bound forms of p62 are less acidic and not sensitive to alkaline phosphatase digestion.

Association of the cytosolic complex with TGN38/41 is essential for formation of exocytic transport vesicles from the TGN. Immunodepletion of the p62 complex from cytosol with antibodies against either p62 or rab6 abolishes the budding of exocytic transport vesicles. The ~10% background

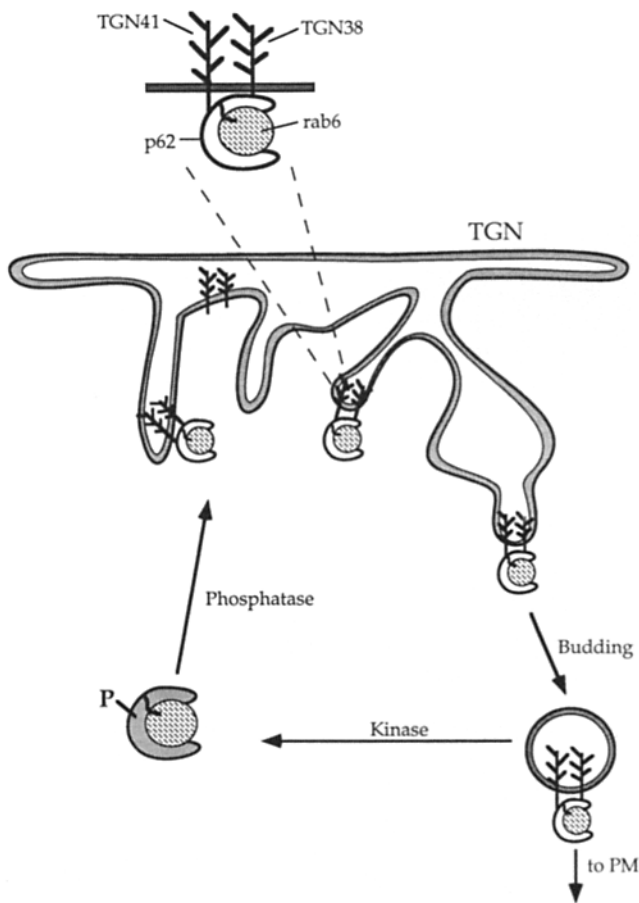


Figure 11. Model of p62 interaction with TGN38/41.

budding which takes place could be carried out by the complexes which are already bound to TGN38/41. Peptides of the COOH terminus of both TGN38 and TGN41 also block the budding process, suggesting they compete with the transmembrane proteins for the binding of the p62 cytosolic complex; whereas a peptide of the NH<sub>2</sub>-terminus of TGN38/41 has no effect on the budding. In addition, antibodies against p62 added to the cytosol are sufficient to abolish the budding. These assays do not allow us to conclude whether p62, rab6, or both are directly taking part in the budding reaction. It is conceivable that p62 delivers rab6 to the appropriate site and rab6 is the functional molecule in budding. Many alternative hypotheses exist; one would be that rab6 functions to proofread the fidelity of the complex formation and p62 is the functional molecule in budding. Since the complex is required for budding and TGN38/41 is moving to the plasma membrane, we assume the complex moves into transport vesicles. Phosphorylation of p62 would dissociate the complex from transport vesicles or even later during fusion of transport vesicles with the plasma membrane. Although the cytosolic p62/rab6 complex is essential for the budding of exocytic transport vesicles from the TGN, other molecules such as coat proteins and trimeric G proteins are probably also required (Stow et al., 1991; Leyte et al., 1992; Pimplikar and Simons, 1993). This data and the data of Schwaninger et al. (1992) provide evidence that rab's with their associated proteins function in vesicle formation.

The p62 complexes present in cytosol and on Golgi mem-

branes were characterized by coimmunoprecipitation, sizing column chromatography, and velocity sedimentation in sucrose gradients. In cytosol, coimmunoprecipitation and 2D-gel analysis show that p62 is associated with four GTP-binding proteins, one of which is rab6. By sizing column chromatography and velocity sedimentation, the cytosolic complex is ~86 kD, indicating that one molecule of p62 associates with only one GTP-binding protein at a time. In contrast, when associated with the Golgi membrane, coimmunoprecipitation and 2D-gel analysis show p62 is associated with any one of three GTP-binding proteins, all but "N" are the same as characterized in the cytosol complex (including rab6) and also with TGN38/41. By sizing column chromatography and velocity sedimentation, the membrane complex is ~250 kD. The simplest stoichiometry for the membrane complex is one copy of the TGN38/41 dimer (160 kD) and one copy of the p62, small GTP-binding protein complex (86 kD). The association of the p62 complex with the membrane is stable to high pH and resistant to dissociation by NP-40 and CHAPS detergents.

We have focused on rab6 because it has been implicated in Golgi function by virtue of being specifically associated with Golgi membranes. More importantly, one of the GTP-binding proteins which coimmunoprecipitates with both p62 and TGN38/41 antibodies was identified as rab6. Lukas Huber at EMBL, Heidelberg, Germany has run these samples in parallel with the 2D mapping studies of the rab proteins he is carrying out. His data confirms that "C" is rab6 and that the GTP-binding proteins labeled "B" and "D" have not been identified (Huber, L., personal communication). 2D-gel analysis shows that some of rab6 in the membrane complex is modified as compared with the form of rab6 in the cytosolic p62 complex. Although we do not know the nature of the modification, it is unlikely to be phosphorylation, as has been shown for rab1A and rab4 (Bailly et al., 1991; van der Sluijs et al., 1992) since the charge shift is more basic rather than more acidic. A modification more consistent with the data would be carboxyl methylation, which has been shown with ras-related proteins during receptor-mediated signal transduction (Philips et al., 1993).

What is the significance of more than one GTP-binding protein associating with the p62 containing complexes both in the cytosol and with TGN38/41? This may mean that more than one GTP-binding protein is loaded onto the membrane for multiple functions, e.g., budding and fusion, and all use the same "loading-protein." Antibodies against these unknown GTP-binding proteins will be required to further dissect the process. Specific antibodies will allow a more precise definition of the stoichiometry of the cytosolic and membrane complexes, providing better insight into the function of each molecule.

Geranylgeranyl modification of the small GTP-binding proteins is likely to play an important role in their interaction with p62 (for review, see Kinsella and Maltese, 1991; Khosravi-Far et al., 1991; Peter et al., 1992). However, since the interaction between p62 and the small GTP-binding proteins is stable in CHAPS, protein-protein interactions must also be predicted.

Under our experimental conditions, using column chromatography or velocity sedimentation in a sucrose gradient, a variable proportion (60–30%) of the TGN38/41 molecules are complexed with p62, rab6, and the other GTP-binding

proteins. The experimental manipulations may have resulted in some dissociation of the p62 complex, or the variability may, in fact, reflect different physiological states.

TGN38/41 is not retained in the TGN but moves into a transport vesicle, and then to the plasma membrane. Its residence on the plasma membrane is short and recycling occurs via the endocytic pathway (Ladinsky and Howell, 1992). The signals on the cytoplasmic domain required for internalization of TGN38/41 have been characterized (Humphrey et al., 1993; Bos et al., 1993). TGN38/41 may function as the receptor which allows the cytosolic complex to participate in the budding process and in so doing winds up in the transport vesicle. Cycling is the mechanism that returns TGN38/41 to the TGN for further rounds of budding. In addition, TGN38/41 may have multiple functions in the vesicular transport process which remain to be characterized.

Because of the tight association of the p62 complex with the membrane, a specific regulator could be predicted to be required for the release of the p62 complex to the cytosol. Phosphorylation has been shown to control membrane association/dissociation of a number of regulatory proteins (van der Sluijs, 1992; Ottaviano and Gerace, 1985). Our data suggest that phosphorylation also regulates the association of the p62 complex with the membrane, as p62 is phosphorylated *in vivo*. More insight comes from the *in vitro* phosphorylation which was carried out using carbonate washed stacked Golgi fraction without addition of cytosol or exogenous kinases. Some of the p62 molecules dissociated from the TGN38/41 complex and those that dissociated were phosphorylated. Dissociation was shown on sucrose velocity gradients where a small proportion of the p62 molecules sedimented at the same velocity as the cytosolic complex rather than the velocity of the membrane complex. Only the dissociated p62 molecules were phosphorylated. Further evidence came from experiments carried out in parallel, where the phosphorylated stacked Golgi fraction was immunoprecipitated with antibodies against both p62 and TGN38/41. Consistent with the sedimentation data, none of the p62 associated with TGN38/41 was phosphorylated. The phosphorylated p62 was immunoprecipitated as an additional, alkaline phosphatase-sensitive band of higher molecular weight only with antibodies against p62. This data is consistent with the alkaline phosphatase sensitivity and the isoelectric forms of p62 characterized by 2D-gel electrophoresis and discussed above.

Our hypothesis is that the cycle of association-dissociation of the p62 complex with TGN38/41 is regulated by phosphorylation. Identification of the specific kinase and phosphatase carrying out these modifications will provide the means to test this hypothesis. The kinase is most likely associated with the Golgi membrane. In fact, kinases already have been shown to be associated with Golgi membranes—both general kinases (Capasso et al., 1985) and specifically cyclic AMP-dependent kinase type II (Nigg et al., 1985) as well as its high affinity binding protein (which binds the regulatory subunit) (Rios et al., 1992). Interestingly, Capasso et al. (1985) showed a 62-kD Golgi protein which was phosphorylated by endogenous kinases and the phosphorylation was stimulated by cAMP. In our *in vivo* experiments, the level of phosphorylation of p62 achieved was not extensive, indicating regulated turnover of the phosphate moiety. In the *in vitro* assay, phosphorylation was more extensive which suggests that the phosphatase is cytosolic.

What kind of a protein is p62? It is clearly a protein which binds small GTP-binding proteins and is necessary for formation of exocytic transport vesicles from the TGN. Could p62 be one of the characterized regulatory proteins of the small GTP-binding proteins: GDP dissociation stimulator (GDS), GDP dissociation inhibitor (GDI), or GTPase activation protein (GAP) (for review see Takai et al., 1992)? These molecules are involved in the GDP/GTP exchange, inhibition of GDP dissociation or activation of GTP hydrolysis, respectively. p62 shares some features of all of these regulatory proteins in that they bind to the GTP-binding protein in the cytosol. We do not know enough about the GTP/GDP status of the GTP-binding proteins associated with p62 to predict a regulatory function. The current consensus is that the GDI delivers the small GTP-binding proteins to a membrane, but then is released and the GDI never actually associates with the membrane itself. p62 functions like a GDI in delivering the small GTP-binding proteins to the membrane but does not appear to dissociate and release the small GTP-binding protein. In fact, p62 is so tightly associated with TGN38/41 that this property allowed its identification. Further characterization of p62, both molecular and biochemical, will define the regulatory role of p62.

In this paper we have shown that the TGN38/41 heterodimer serves as a "receptor" for a cytosolic complex containing p62, rab6, or one of two other unidentified small GTP-binding proteins. This interaction is essential for budding of exocytic transport vesicles from the TGN. TGN38/41 can be envisioned to have an even more extensive role in the budding process. This transmembrane receptor could function as a sorting molecule by interaction of soluble luminal proteins with its luminal domain, signaling the initiation of exocytic vesicle formation.

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