A GDP-Bound Form of Rab1 Inhibits Protein Export from the Endoplasmic Reticulum and Transport between Golgi Compartments

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Abstract. Rab1 is a small GTPase regulating vesicular traffic between early compartments of the secretory pathway. To explore the role of rab1 we have analyzed the function of a mutant (rab1a[S25N]) containing a substitution which perturbs Mg^{2+} coordination and reduces the affinity for GTP, resulting in a form which is likely to be restricted to the GDP-bound state. The rab1a(S25N) mutant led to a marked reduction in protein export from the ER in vivo and in vitro, indicating that a guanine nucleotide exchange protein (GEP) is critical for the recruitment of rab1 during vesicle budding. The mutant protein required posttranslational isoprenylation for inhibition and behaved as a competitive inhibitor of wild-type rab1 function. Both rab1a and rab1b (92% identity) were able to antagonize the inhibitory activity of the rab1a(S25N) mutant, suggesting that these two isoforms are functionally interchangeable. The rab1 mutant also inhibited transport between Golgi compartments and resulted in an apparent loss of the Golgi apparatus, suggesting that Golgi integrity is coupled to rab1 function in vesicular traffic.

MEMBERS of the *rab/YPT1/SEC4* family of ras-related GTPases are associated with distinct subcellular compartments comprising the endocytic and exocytic pathways (for review see Zerial and Stenmark, 1993; Nuoffer and Balch, 1994). Although their precise functions are unknown, these proteins are likely to serve as molecular switches which regulate the assembly/disassembly of protein complexes involved in the targeting and fusion of transport vesicles mediating the vectorial transfer of protein between secretory compartments. In yeast, Ypt1p was the first small GTPase shown to be required for vesicular traffic between the ER and the Golgi compartments (for review see Pryer et al., 1992; Ferro-Novick and Novick, 1993). The rab1a and rab1b proteins (92% identical) are 75 and 66% identical to Ypt1p, respectively, and are considered the mammalian counterparts of Ypt1p (Touchot et al., 1987). Overexpression of mouse rab1a can rescue yeast mutants lacking Ypt1p function (Haubruck et al., 1989). Furthermore, several lines of evidence now suggest that rab1 is essential for both ER to Golgi and intra-Golgi transport in mammalian cells in vivo and in vitro (Plutner et al., 1990, 1991; Tisdale et al., 1992; Davidson and Balch, 1993).

GTPases go through a characteristic cycle of reactions which drive the transition between at least two distinct conformational states (for review see Bourne et al., 1990, 1991; Wittinghofer and Pai, 1991). Release of GDP from the 'inactive' (GDP-bound) state allows binding of GTP. This converts the protein to the 'active' (GTP-bound) form. It then returns to the inactive state upon GTP hydrolysis, an irreversible reaction which renders the cycle unidirectional. The function of individual GTPases as molecular switches depends on the abilities of these distinct conformational states to interact with specific macromolecules. These include putative 'effector' proteins and various regulatory factors which control the GTPase cycle, such as guanine nucleotide dissociation inhibitors (GDI)[1] which inhibit GDP dissociation, guanine nucleotide exchange proteins (GEP) which stimulate GDP dissociation, and GTPase-activating proteins (GAP) which promote GTP hydrolysis (for review see Takai et al., 1992; Nuoffer and Balch, 1994).

All members of the GTPase superfamily share a number of highly conserved sequence motifs critical for guanine nucleotide binding and GTP hydrolysis (Bourne et al., 1991). Extensive mutational analysis of these domains in the case of H-ras have led to the identification of numerous substitutions which alter the affinity for guanine nucleotides and/or prevent GTP hydrolysis (for review see Barbacid, 1987; Lowy and Willumsen, 1993). These mutations are believed to restrict the protein to either the inactive, GDP-

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1. *Abbreviations used in this paper*: BFA, brefeldin A; endo-H, endoglycosidase H; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEP, guanine nucleotide exchange protein; GGPP, [^{3}H] geranylgeranyl pyrophosphate; Mann II, α-1,2-mannosidase II; VSV-G, vesicular stomatitis virus glycoprotein.

bound or the active, GTP-bound state and interfere with normal H-ras function in a dominant fashion.

We previously screened a number of rabl mutants to identify substitutions which inhibit ER to Golgi traffic in vivo (Tisdale et al., 1992). We now analyze in detail the biochemical properties and the inhibitory phenotype of rabla(S25N), a mutant analogous to the growth inhibitory H-ras(S17N) mutant which appears to be restricted to the GDP-bound conformation and has been proposed to interfere with the function of ras-GEP (Feig and Cooper, 1988; Farnsworth and Feig, 1991). Our data show that the S25N substitution in rabl reduces the affinity for GTP by 100-fold, supporting the view that the mutant is restricted to the inactive, GDP-bound form. We find that the S25N mutant perturbs transport by inhibiting protein export from the ER by a mechanism that depends on posttranslational isoprenylation and involves competition with wild-type rabl. The mutant also inhibits transport between Golgi compartments and disrupts the morphological integrity of the Golgi complex. These studies suggest a cycle in which the function of a rabl-specific GEP mediating guanine nucleotide exchange is critical for the recruitment of rabl, and the formation of ER and Golgi carrier vesicles competent for fusion with downstream compartments.

Materials and Methods

Materials

A polyclonal serum recognizing α-1,2-mannosidase II (anti-Man II) was prepared as described (Velasco et al., 1993). A monoclonal cell line producing antibodies which recognize the cytoplasmic tail of vesicular stomatitis virus glycoprotein (VSV-G) (P5D4) was provided by K. Howell (University of Colorado, Denver, CO). A polyclonal reagent recognizing p53 was provided by H.-P. Hauri (Biocenter, Basel, Switzerland). Secondary antibodies were obtained from the following sources: FITC-conjugated goat anti-rabbit $F(ab)_2$ and anti-mouse IgG from Zymed Labs. (S. San Francisco, CA).

Generation of Mutant Constructs

The construction of the rablb(S22N) mutant has been described previously (Tisdale et al., 1992). New mutations were created using the PCR and appropriate subcloning strategies. All constructions were introduced into the NdeI and BamHI sites of pET3a for expression under control of the phage T7 promoter. The S25N, T43A, and D47N substitutions were introduced into canine rabla (Chavrier et al., 1990) using a two-step PCR procedure involving two complementary mutagenic oligonucleotides in combination with flanking 5′ and 3′ primers. In a first set of reactions, overlapping 3′ and 5′ fragments were generated using pET3a-rabla (Khosravi-Far et al., 1992; Tisdale et al., 1992) as a template. The T7 primer (5′-TTAATACGACTCACTATAGG-3′) and the anti-sense mutagenic oligonucleotides 5′-CTAAGAAGGAGGCAATTCTTTCCAACCCC-3′ (S25N substitution, anticodon for Asn-25 underlined), 5′-ATCCACACCAATTGCGCTGATGTAGC-3′ (T43A), and 5′-TCTTATTTTGAAATTCACACCAATTGT-3′ (D47N), served as primers for amplification of the 5′ fragments. The 3′ fragments were obtained using oligonucleotides complementary to the above mutagenic primers in combination with the 3′ anti-sense primer 5′-TAGGATCCGCTAGCTTAGCAGCAACCTCCACCTGA-3′. These fragments were combined to generate the full-length mutant sequence in a second reaction using the T7 and 3′ anti-sense primers. For amplification of His_6-tagged rabla constructs, the T7 primer was replaced by the oligonucleotide 5′-TAATCGATCATATGCATCATCATCATCATCATTCCAGCATGAATCCCGAA-3′ (His codons underlined) The S25N double-mutants were obtained by successive rounds of mutagenesis. The rabla(ΔCC) truncation was constructed using the 3′ anti-sense oligonucleotide 5′-GTGGATCCCTCGAGTTAACCTCCACCTGACTGCTTG-3′ (new termination codon underlined) and introduced into the S25N mutant by exchange of the unique EcoRI fragment common to pET3a-rabla plasmids. The 3′ anti-sense primer 5′-GGGGGATCCTATCGCTTTTTAATCTCAGC-3′ (new termination codon underlined) was used for generation of the Δ176-205 deletion using pET3a-rabla(N124I) as a template. His_6-tagged forms of wild-type rablb and rablb(S22N) were generated from the existing pET3a constructs (Tisdale et al., 1992) using the oligonucleotides 5′-TAATCGATCATATGCATCATCATCATCATCATAACCCCGAATATGACTAC-3′ (5′ sense primer, His codons underlined) and 5′-TAGGATCCGCTAGCTTAGCAGCAGCCACCACTAGC-3′ (3′ anti-sense primer). All PCR products were examined by DNA sequencing to confirm the mutations and exclude other changes. A His_6-tagged form of rab3a was constructed by transferring the rab3a sequence as an NdeI–BamHI fragment from pET3a-rab3a (Tisdale et al., 1992) simultaneously with a NcoI-NdeI linker encoding an initiator Met, a Gly, and six consecutive His residues (assembled using the overlapping complementary oligonucleotides 5′-CATGGGCCATCATCATCATCATCA-3′ [sense primer, Met codon underlined] and 5′-TATGATGATGATGATGATGGCC-3′ [anti-sense primer]), into the NcoI and BamHI sites of pET11d (Novagen, Madison, WI).

Transient Expression and Analysis of Transport in HeLa Cells

Experimental procedures for the transient expression of pET-rab constructs in HeLa cells were essentially as described previously (Tisdale et al., 1992). Briefly, cells infected with the T7 RNA polymerase-recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986) were cotransfected with 1 μg pAR-G (encoding VSV-G),and 2.5 μg of the appropriate pET-rab constructs using the TransfectACE™ (GIBCO-BRL, Gaithersburg, MD) procedure. After pulse-labeling the cells for 10 min with 20 μCi Trans^{35}S-label (1192 Ci/mmol, ICN Biomedicals Inc., Irvine, CA) followed by a 60-min chase, transport between the ER and the cis/medial Golgi compartments was assessed biochemically by monitoring the processing of VSV-G from endoglycosidase H (endo H)-sensitive to endo H-resistant forms as described (Tisdale et al., 1992), except for the following modifications: the monoclonal anti-VSV-G antibodies (clone 8G5, 5 μl/0.5 ml lysate) were added without preclearing the lysates. After incubation at 4°C overnight, 40 μl of a 1:1 suspension of protein A-Sepharose™ (Sigma Chem. Co., St. Louis, MO) in TBST (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-100 (Surfact-Amps™, Pierce Chem. Corp., Rockford, IL)) were added, and the samples incubated for 1 h at room temperature.

Immunoprecipitates were washed 3× with TBST, 1× with 10 mM Tris-HCl (pH 6.8), boiled, and the eluates precipitated with 0.5 ml acetone for 15 min on ice. The pellets were resuspended in 20 μl 0.1 M NaOAc (pH 5.6), 0.3% Na-dodecyl sulfate (SDS) and 2% β-mercaptoethanol, boiled, diluted with 40 μl 0.1 M NaOAc (pH 5.6), and digested with 5 mU endo H (Boehringer Mannheim Corp., Indianapolis, IN) at 37°C overnight. The samples were processed for SDS-PAGE and fluorography as described (Tisdale et al., 1992). Autoradiographs were quantitated by scanning densitometry (GS300 transmission scanning densitometer, HOEFER Scientific Instruments, San Francisco, CA). For morphological analysis of transport, cells cotransfected with pAR-tsO45-G (encoding the temperature-sensitive tsO45 mutant of VSV-G [Lafay, 1974]), and appropriate pET-rab constructs were incubated at the restrictive temperature (39.5°C) for 4 h to accumulate the protein in the ER. Transport was initiated by shifting the cells to the permissive temperature (32°C). After a 2-h incubation the cells were processed for indirect immunofluorescence as described (Tisdale et al., 1992).

Expression and Purification of His_6-Tagged Proteins

Plasmids were introduced into *E. coli* strain BL21(DE3)pLysS (Novagen) for expression. After induction with 0.4 mM isopropyl-β-thiogalactopyranoside for 1–4 h at 28°, the cells were collected, resuspended in 10 vol buffer A (50 mM Tris-HCl (pH 8.0) containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 μM GDP, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin A), and lysed by digestion with lysozyme (400 μg/ml) for 30 min at 4°C and two cycles of freeze-thawing. The cell lysate was adjusted to 300 mM NaCl, 10 mM $MgCl_2$, 0.2% deoxycholate (final concentrations), and incubated with DNAseI (40 μg/ml) for 30 min at 4°C. After centrifugation (22,000 *g* for 30 min), the supernatant was applied to a 2.5-ml column of nickel-saturated nitrilotriacetic acid (NTA)-agarose (Qiagen, Chatsworth, CA) equilibrated with buffer A containing 300 mM NaCl and 10 mM $MgCl_2$. The column was washed with 10 vol each of the equilibration buffer, buffer B (50 mM Mes-NaOH (pH 6.0) containing 300 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 50 μM EGTA, and 1 μM GDP), and buffer B supplemented

with 25 mM imidazole. The column was then developed with buffer B containing 250 mM imidazole, and 2-ml fractions collected. Fractions containing rabla were pooled and applied to a 150 × 1.6 cm column of Sephacryl S-100 equilibrated with 25 mM Hepes-KOH (pH 7.2), containing 125 mM KOAc, 1 mM $MgCl_2$, 1 mM Na mercaptoethane sulphonic acid, and 1 μM GDP. Peak fractions containing rabla were pooled, concentrated by ultrafiltration and stored in aliquots at −70°C. Protein concentrations were determined with the Coomassie Plus™ reagent (Pierce Chem. Co.) using BSA as a standard.

Guanine Nucleotide Exchange Reactions

Guanine nucleotide exchange reactions were routinely performed in duplicate using 0.5 μg (~20 pmol) recombinant rab protein in 100 μl 50 mM Hepes-KOH (pH 8.0), 1 mM DTT, 0.1 mg/ml BSA, 5 mM EDTA, 4.5 mM $MgCl_2$, and 2.5 μM [^{3}H]GDP (25–50 Ci/mmol; New England Nuclear Corp., Wilmington, DE; diluted to ~5,000 cpm/pmol with unlabeled GDP). For competition experiments, reactions were supplemented with unlabeled nucleotides as specified. The samples were incubated at 32°C for the desired period of time, transferred to ice, diluted with 2 ml of an ice-cold buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 30 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, and immediately filtered through prewet 0.45 μm pore-size nitrocellulose filter discs (BA85, Schleicher and Schuell, Keene, NH). The tubes were rinsed 1× with 2 ml dilution buffer, filters washed 3× with 2 ml dilution buffer without DTT and BSA, and protein-bound [^{3}H]GDP was quantitated by liquid scintillation counting.

Prenylation of Recombinant Proteins In Vitro

To assess posttranslational isoprenylation of recombinant rab proteins in vitro, 26 pmol [^{3}H]geranylgeranyl pyrophosphate (GGPP) (19.3 Ci/mmol, New England Nuclear Corp.) were dried in a speed-vac lyophilizer and resuspended in a final volume of 50 μl containing 1 μg (~40 pmol) recombinant protein, 25 μl cytosol (5–15 μg/ml protein in 25 mM Hepes-KOH (pH 7.2), 125 mM KOAc) prepared from rat liver homogenates as described (Davidson et al., 1992), 10 mM $MgCl_2$, 1 mM MesNa, and an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU rabbit muscle creatinine phosphokinase, final concentrations). After incubation for 1 h at 32°C, the samples were boiled in sample buffer (0.5 M Tris-HCl (pH 6.8), 0.1% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% Bromphenol blue, final concentrations) and analyzed by SDS-PAGE on 12% gels. The gels were soaked in 1 M salicylate for 1 h, dried, and exposed to XAR-5 film (Eastman Kodak Corp., Rochester, NY) at −70°C.

For subsequent inclusion of posttranslationally processed proteins into in vitro transport reactions, 5 μg recombinant rabla were preincubated as outlined above, except that 10 μg (20 nmol) of unlabeled GGPP (generously provided by A.M. Garcia [Eisai Research Institute, Andover, MA]) (10 μl of a 1-mg/ml solution in 25 mM Hepes-KOH [pH 7.2], 125 mM KOAc) were substituted for the radiolabeled precursor. Since the composition of the prenylation cocktail was incompatible with the conditions required for efficient transport, the samples were "desalted" by centrifugation (10 min at 1,500 *g*) through 1 ml Sephadex™ G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) columns equilibrated with 25 mM Hepes-KOH (pH 7.2), 125 mM KOAc before further usage. The efficiency of the prenylation reaction was estimated by phase separation in Triton X-114 solution as described (Bordier, 1981). Briefly, the samples were adjusted to ~1% Triton X-114 on ice, and phases were separated by incubation at 32°C followed by centrifugation through a sucrose cushion. The aqueous phase was reextracted with ~0.5% Triton X-114, phases were adjusted to equal volumes, and proteins precipitated with TCA. The pellets were washed with acetone, resuspended in sample buffer, boiled, and analyzed by SDS-PAGE on 12% gels followed by Western blotting using anti-rabla antibodies essentially as described (Plutner et al., 1991; Tisdale et al., 1992). Using recombinant rabla as a standard, we estimated that 250–500 ng of rabla were routinely recovered in the detergent phase upon incubation in the presence of GGPP, suggesting that 5–10% of the protein is processed under these conditions. No processing could be detected in the absence of exogenous GGPP.

Analysis of Transport In Vitro

NRK cells were infected with the tsO45 strain of VSV and pulse-labeled with 100 μCi Trans ^{35}S-label at the restrictive temperature (39.5°C) to accumulate the VSV-G mutant in the ER. The cells were then perforated by the swelling and scraping procedure as described (Beckers et al., 1987). Transport between the ER and the cis-medial Golgi compartments was measured biochemically by following the appearance of endo H-resistant forms of VSV-G upon incubation at the permissive temperature (32°C) in the presence of cytosol and ATP as described previously (Davidson et al., 1992; Schwaninger et al., 1991). Briefly, transport reactions were performed in a final volume of 40 μl in a buffer containing 25 mM Hepes-KOH (pH 7.2), 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM N-acetyl glucosamine, an ATP-regenerating system (1 mM ATP, 5 mM creatinine phosphate, and 0.2 IU rabbit muscle creatine phosphokinase, final concentrations), 2–6 μl rat liver cytosol (20–60 μg/ml protein in 25 mM Hepes-KOH (pH 7.2), 125 mM KOAc), and 5 μl semi-intact cells (25–50 μg protein or 1–2 × 10^5 cells in 50 mM Hepes-KOH [pH 7.2], 90 mM KOAc). Recombinant proteins were preincubated with rat liver cytosol in the presence or absence of GGPP, and the samples desalted as outlined above. Aliquots of these reactions were then added to the transport cocktails together with the minimal volume of fresh cytosol required for efficient transport under standard conditions. The reactions were incubated at 32°C for 2 h, membranes collected by centrifugation, solubilized, digested with endo H, and processed for SDS-PAGE and fluorography as described (Beckers et al., 1987). Autoradiographs were quantitated by scanning densitometry. For morphological analysis of transport, the cells were permeabilized with digitonin as described (Plutner et al., 1992). Incubation conditions were as outlined above, except that the reactions were performed in a final volume of 200 μl. The cells were processed for indirect immunofluorescence as described previously (Plutner et al., 1992).

Microinjection of Cells with Recombinant Proteins

NRK cells were infected with the tsO45 strain of VSV at the restrictive temperature as described (Plutner et al., 1992). The cells were then microinjected with an IgG marker and recombinant wild-type or mutant rabla as described (Wilson et al. 1994). After a 1.5-h incubation at the restrictive temperature, transport was initiated by shifting the cells to the permissive temperature. The cells were processed for immunofluorescence as described previously (Plutner et al., 1992).

Results

Guanine Nucleotide Binding Properties of Recombinant Rabla(S25N)

To focus on the role of guanine nucleotide exchange in rabl function, we generated the rabla(S25N) mutant which contains a substitution in the GxxxxGKS/T motif (residues 10-17 in H-ras) involved in phosphate binding and Mg^{2+} coordination (Pai et al., 1989, 1990). It is analogous to the dominant negative H-ras(S17N) mutant, which inhibits cell proliferation and appears to be restricted to an inactive, GDP-bound state (Farnsworth and Feig, 1991; Feig and Cooper, 1988). To establish the guanine nucleotide-binding properties of the rabla(S25N) mutant, we purified the protein after overexpression in *E. coli*, taking advantage of an amino-terminal His_6-modification (data not shown) (Hochuli et al., 1988). When transiently expressed in vivo, His_6-tagged wild-type and mutant proteins have properties identical to their untagged counterparts (data not shown). After expression in *E. coli*, ras-like GTPases are normally isolated as stable GDP-protein complexes (Tucker et al., 1986). In the presence of high concentrations of free Mg^{2+}, the protein-bound nucleotide is only slowly displaced by exogenously added GDP or GTP, a reaction which can be accelerated by complexing Mg^{2+} with EDTA (Hall and Self, 1986). During incubation of recombinant wild-type rabla in the presence of [^{3}H]GDP and a low concentration of free Mg^{2+} (5 mM EDTA, 4.5 mM Mg^{2+}), the endogenous GDP was readily exchanged for the radiolabeled nucleotide (Fig. 1 *A*; *left panel, open circles*). No exchange was observed at a high concentration of free Mg^{2+} (5 mM EDTA, 10 mM Mg^{2+}) (Fig. 1 *A*, *right panel*, compare lanes *a* and *b*). Anal-

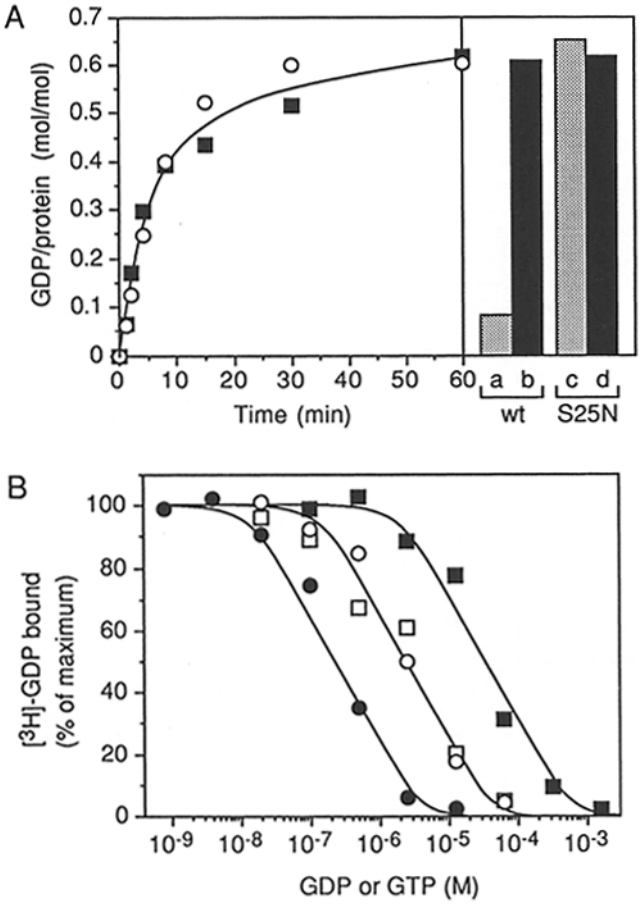


Figure 1. The rabla(S25N) mutant shows preferential affinity for GDP. (*A*) Time course and Mg^{2+} dependence of GDP exchange. Exchange reactions were performed in duplicate by incubating 0.5 μg (20 pmol) of recombinant protein at 32°C with 2.5 μM [^{3}H]GDP (~5,000 cpm/pmol) in the presence of EDTA and $MgCl_2$. Bound [^{3}H]GDP was quantitated by liquid scintillation counting after capture of the proteins on nitrocellulose membranes as described in Materials and Methods. (*Left panel*) The wild-type (○) and mutant proteins (■) were incubated for the specified times with [^{3}H]GDP in the presence of 5 mM EDTA and 4.5 mM $MgCl_2$, and the amount of protein-bound GDP was determined for each time point. (*Right panel*) The wild-type (*wt*, lanes *a* and *b*) and mutant proteins (*S25N*, lanes *c* and *d*) were incubated for 1 h with [^{3}H]GDP in the presence of 5 mM EDTA and either 10 mM $MgCl_2$ (*gray bars*, lanes *a* and *c*) or 4.5 mM $MgCl_2$ (*black bars*, lanes *b* and *d*), and the amount of protein-bound GDP was determined. (*B*) Relative affinities of the proteins for GDP and GTP. The wild-type (○, ●) and mutant (□,■) proteins were incubated for 1 h with [^{3}H]GDP in the presence of 5 mM EDTA and 4.5 mM $MgCl_2$, and the indicated concentrations of unlabeled GDP (○,□) or GTP (●,■). The results are expressed as the percentage of the amount of [^{3}H]GDP bound in the absence of additional nucleotide.

ysis of the rabla(S25N) mutant showed that the rate and extent of exchange were similar to those of the wild-type protein (Fig. 1 *A*, *left panel*, *closed squares*). In this case, however, high concentrations of free Mg^{2+} did not stabilize the protein-bound nucleotide (Fig. 1 *A*, *right panel*, compare lanes *c* and *d*), consistent with structural data suggesting a role of the equivalent residue in H-ras in Mg^{2+} coordination (Farnsworth and Feig, 1991; Pai et al., 1989, 1990).

Competition experiments were performed to compare the affinities of wild-type rabla and the rabla(S25N) mutant for GDP and GTP. The recombinant proteins were incubated at low Mg^{2+} concentration with [^{3}H]GDP (2.5×10^{-6} M) in the presence of increasing concentrations of unlabeled GDP (Fig. 1 *B*, *open symbols*) or GTP (Fig. 1 *B*, *closed symbols*). In the case of the wild-type protein, supplementing the reaction with ~2×10^{-7} M GTP was sufficient to reduce [^{3}H]GDP binding by ~50% (Fig. 1 *B*, *closed circles*). Since this value is ~10-fold *less* than the GDP concentration present in the reaction, it appears that wild-type rabla has a higher affinity for GTP than for GDP. In contrast, a ~10-fold *excess* of GTP was necessary for half-maximal inhibition of [^{3}H]GDP binding to the rabla(S25N) mutant (Fig. 1 *B*, *closed squares*). These data establish that the S25N substitution reduces the affinity of rabla for GTP without altering its affinity for GDP.

Rabla(S25N) Must be Geranylgeranylated to Inhibit Transport In Vivo

Having established that the rabla(S25N) mutant is likely to be restricted to the GDP-bound conformation, we examined its effect on transport between the ER and the Golgi compartment in vivo using a vaccinia virus-dependent transient expression system (Tisdale et al., 1992). HeLa cells infected with the T7 RNA polymerase-recombinant virus were cotransfected with plasmids encoding the appropriate rab construct(s) and the VSV-G under control of the phage T7 promotor. VSV-G is a type I integral membrane protein which acquires two N-linked oligosaccharides during cotranslational insertion into the ER membrane (Etchison et al., 1977). It is processed through a transient endo H-resistant 'R_I' form in the cis/medial Golgi compartments, before acquisition of terminal sialic acids in the *trans*-Golgi to form the mature 'R_T' form (Schwaninger et al., 1991; Plutner et al., 1992; Tisdale et al., 1992; Davidson and Balch, 1993). These carbohydrate processing intermediates can be resolved by their unique mobilities using SDS-PAGE (Fig. 2, lane *b*).

As shown in Fig. 2 (lane *c*), transient expression of the rabla(S25N) mutant potently inhibited ER to Golgi transport. In this case, only ~10% of the VSV-G was processed to the R_I form (Fig. 2, lane *c*). In this and subsequent experiments, the expression level of the various rabl wild-type and mutant proteins was between 2- and 8-fold over the endogenous pool (data not shown). The equivalent mutation in the rablb isoform (rablb(S22N)) also inhibits transport (Tisdale et al., 1992). Given that the two isoforms have markedly different carboxyl-terminal sequences which appear to be involved in membrane localization (Chavrier et al., 1991), the two proteins may be targeted to different components.

We have consistently noticed that the small fraction of VSV-G (generally 5–15%) which is processed to the R_I form in the presence of the mutant is not further modified to the R_T form (Fig. 2, lane *c*). Processing to the latter form is diagnostic of transport from the cis to the trans Golgi compartments (Davidson and Balch, 1993). These data suggest that the rabla(S25N) mutant not only inhibits ER to Golgi transport, but also transport through compartments of the Golgi stack and supports our previous observation that a neutralizing, rabl-specific antibody inhibits both ER to Golgi and intra-Golgi transport (Plutner et al., 1991).

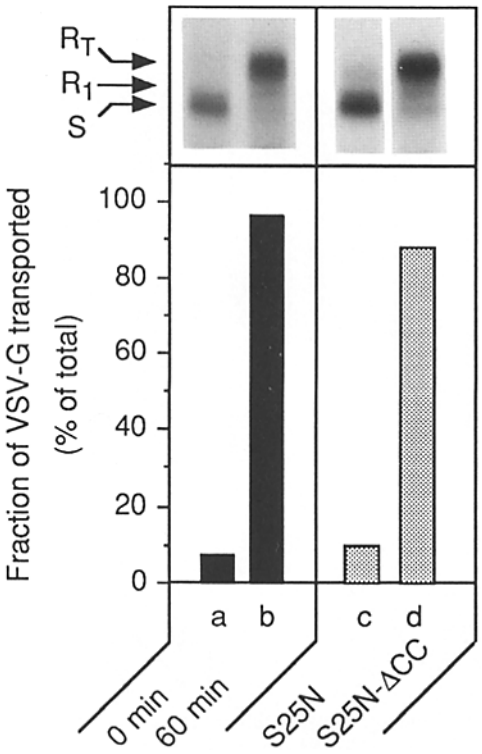


Figure 2. The S25N mutant requires prenylation for function in vivo. HeLa cells were infected with the T7 RNA polymerase-recombinant vaccinia virus vTF7-3 and cotransfected with plasmids encoding VSV-G (lanes *a* and *b*) and the indicated rabla constructs (lanes *c* and *d*) as described in Materials and Methods. After 5 h, cells were pulse-labeled with Trans ^{35}S-label for 10 min (lane *a*), chased for 60 min (lanes *b–d*), VSV-G was immunoprecipitated, digested with endo H, and the endo H-sensitive (*S*), the endo H-resistant intermediate (R_I) and the terminally glycosylated (R_T) forms were separated by SDS-PAGE, and autoradiographs (*upper panel*) quantitated by scanning densitometry as described in Materials and Methods (*lower panel*). Transport is expressed as the percentage of total VSV-G converted to endo H-resistant forms.

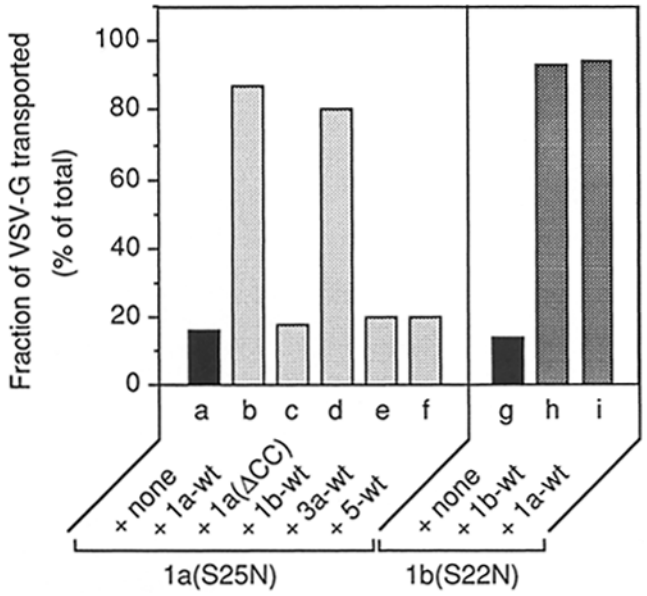


Figure 3. Rabla and rablb isoforms reverse inhibition of the S25N mutant in vivo. The rabla(S25N) and rablb(S22N) mutants were transiently expressed in HeLa cells alone (lanes *a* and *g*) or in combination with the indicated mutant or wild-type (*wt*) rab proteins (lanes *b–f*, *h* and *i*). Their effects on transport of VSV-G between the ER and the Golgi compartments were quantitated as outlined in the legend to Fig. 2 and in the Materials and Methods. Lanes *b* and *c* contrast the abilities of wild-type rabla (lane *b*) or rabla(ΔCC) (lane *c*) to antagonize the inhibitory phenotype of the S25N mutant (lane *a*). Lane *d* shows the consequence of coexpressing the mutant protein with wild-type rablb. The results of the reciprocal experiments using the analogous rablb(S22N) mutant are illustrated in lanes *g–i*.

Several lines of evidence indicate that posttranslational isoprenylation of carboxyl-terminal Cys residues is critical for the function of ras and ras-like GTPases (for review see Der and Cox, 1991). Rabl is normally modified by the addition of two geranylgeranyl (GG) groups to the terminal CC motif (Khosravi-Far et al., 1992). To investigate the role of isoprenylation in rabl function, we determined the phenotype of a truncated form of the rabla(S25N) mutant lacking the terminal Cys residues (rabla[S25N-ΔCC]). As shown in Fig. 2 (lane *d*), deletion of the CC motif eliminated the inhibitory activity of the S25N mutant. In this case, VSV-G was efficiently processed to the intermediate (R_I) and terminally glycosylated (R_T) endo H-resistant forms (Fig. 2, lane *d*), indicating that prenyl groups are likely to be critical to promote interaction of the mutant with the transport machinery.

Both Rabla and Rablb Are Able to Antagonize Rabla(S25N)

To provide insight into the molecular mechanisms underlying the inhibitory effect of the rabla(S25N) mutant, we examined whether the simultaneous overexpression of wild-type rabl might reverse the mutant phenotype. Coexpression of the mutant protein with wild-type rabla (Fig. 3, lane *b*), but not with rabla(ΔCC), which lacks the carboxyl-terminal Cys residues (Fig. 3, lane *c*), could overcome the inhibition. These data not only indicate a competitive relationship between the S25N mutant and the wild-type protein, but provide evidence that isoprenylation is essential for the normal function of rabla. Moreover, these results provided us with an assay to examine directly the functional relationship of rabla and rablb by testing whether one or both isoforms could antagonize the inhibitory effect of the S25N mutant. As shown in Fig. 3, both wild-type rabla and rablb reversed inhibition by either the rabla(S25N) (Fig. 3, lanes *b* and *d*) or rablb(S22N) (Fig. 3, *h* and *i*) mutants at similar levels of expression (data not shown). Thus, despite their divergent carboxyl-termini, it is evident that they may interact with a common target. In contrast, coexpression of the rabla(S25N) mutant with either rab3a, which is believed to regulate the fusion of synaptic vesicles in the nerve terminal (Fischer von Mollard et al., 1990, 1991) or rab5, which is involved in the endocytic pathway (Bucci et al., 1992; Gorvel et al., 1991), at levels similar to those of the rabla and rablb wild-type proteins, did not restore transport (Fig. 3, lanes *e* and *f*). The latter results eliminate the trivial possibility that reversal of inhibition might be due to indirect effects of the coexpression procedure.

Characterization of the Role of the Effector Domain in Rabla Function

To extend our molecular characterization of the functional domains involved in rabl function, we examined mutations in the putative effector domain. The effector domain of ras (residues 32-40 of H-ras) is one of several domains which undergo prominent changes in conformation depending on the phosphorylation state of the bound nucleotide (Wittinghofer and Pai, 1991). Mutations in the effector domain (e.g., T35A) can neutralize the transforming potential of oncogenic forms of ras (Haubruck and McCormick, 1991; Sigal et al., 1986) and often impair the ability of the protein to respond to GAP (Adari et al., 1988; Calès et al., 1988). More recently, mutations in the Yptlp and rab3a effector domains have been reported to render these proteins insensitive to GAP (Becker et al., 1991; Burstein et al., 1992), and, in the case of rab3a, insensitive to GEP (Burstein et al., 1992).

To determine whether analogous amino acids in the effector domain of rabl contribute to function, we examined the consequences of replacing the highly conserved Thr at position 43 of rabla (position 35 in H-ras) with Ala in the rabla wild-type and the S25N mutant. Transient expression of the rabla(T43A) mutant had no effect on ER to Golgi transport (Fig. 4, lane *a*). Moreover, since the rabla(T43N) was able to antagonize the inhibitory phenotype of the S25N mutant (Fig. 4, lane *d*), it is apparent that the rabla(T43N) mutant has normal function and therefore differs significantly from H-ras with respect to the biological role of the highly conserved Thr residue.

A D44N substitution in rablb has recently been suggested to inhibit prenylation (Wilson and Maltese, 1993). In addition, the analogous mutation (D44N) in Yptlp results in a recessive, temperature-sensitive phenotype (Becker et al., 1991). In contrast, we found no inhibitory phenotype at either 37°C (Fig. 4, lane *e*) or 39.5°C (data not shown) in cells overexpressing the analogous rabla(D47N) mutant. Moreover, overexpression of rabla(D47N) was also able to prevent the transport block imposed by the S25N mutant in a fashion comparable to wild-type rabl (Fig. 4, lane *f*). Interestingly, introduction of the D47N substitution into the effector domain of the S25N mutant completely abolished the inhibitory phenotype of the mutant protein (data not shown). Thus, while the D47N substitution does not seem to alter the functional properties of wild-type rabla, it appears to have a significant effect on the capacity of the S25N mutant to interact with its target.

Morphological Analysis of the Inhibitory Effects of the Rabla(S25N) Mutant on VSV-G Transport

To establish the morphological phenotype of the rabla-(S25N) mutant in vivo, a temperature-sensitive mutant of VSV-G (tsO45) was substituted for wild-type VSV-G as a reporter molecule. TsO45 VSV-G has a thermoreversible folding defect and is retained in the ER when HeLa cells are transfected at the restrictive temperature (39.5°C) as indicated by a diffuse immunofluorescence staining pattern (Tisdale et al., 1992). Incubation of transfected cells at the permissive temperature (32°C) results in transport of VSV-G to punctate, perinuclear Golgi structures (Tisdale et al., 1992). In contrast, in the presence of the rabla(S25N) mutant VSV-G largely remained in a diffuse reticular distribution during incubation at the permissive temperature, although in a few cells VSV-G could also be detected in a distribution which overlapped with p53, a marker protein for pre-Golgi transport intermediates (Schweizer et al., 1988) (data not shown). In addition to the transport block, we also noticed that the Golgi complex of cells overexpressing the S25N mutant was considerably more fragmented when compared to cells expressing the wild-type protein (data not shown). This raised the possibility that inhibition of transport by rabla(S25N) might also perturb Golgi structure.

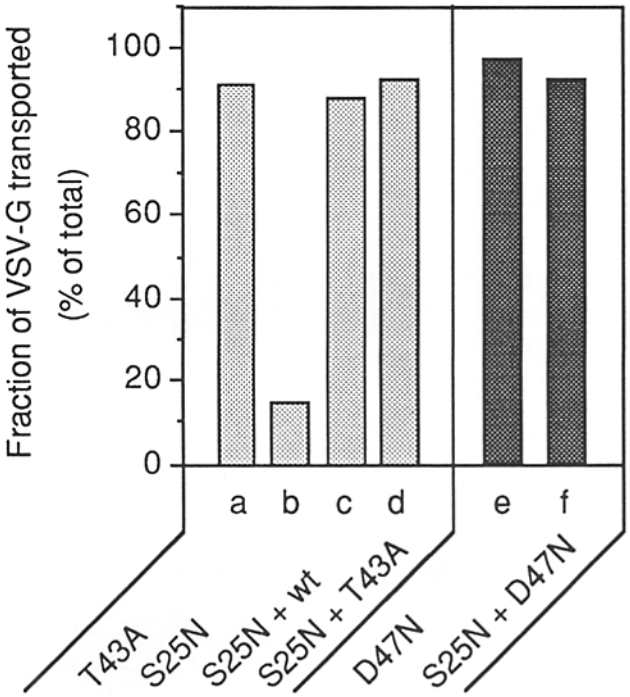


Figure 4. Analysis of effector domain substitutions. The specified rabla single-mutants (lanes *a*, *b*, and *e*) and combinations of mutant and wild-type (*wt*) proteins (*c*, *d*, and *f*) were transiently expressed in HeLa cells. Their effects on transport of VSV-G between the ER and the Golgi compartments were quantitated as outlined in the legend to Fig. 2 and in Materials and Methods. The transport phenotypes of cells expressing the T43A and D47N effector domain mutants alone are illustrated in lanes *a* and *e*, respectively. The ability of wild-type rabla, and the T43A or D47N mutants to antagonize S25N inhibition is shown in lanes *c*, *d*, and *f*, respectively.

We examined more carefully the relationship between inhibition of vesicular traffic and disruption of Golgi structure by microinjecting recombinant rabla(S25N) into NRK cells expressing tsO45 VSV-G. To facilitate efficient prenylation of the recombinant protein in vivo, injected cells were preincubated for 1.5 h at the restrictive temperature before transfer to the permissive temperature for 1.5 h. As shown in Fig. 5, *a–c*, cells microinjected with wild-type rabl (which were identified by coinjection with an IgG marker [*large arrowheads*]) showed migration of VSV-G to typical Golgi complexes as shown by overlap with α-1,2-mannosidase II (Man II), a marker protein localized to the cis/medial compartments (Velasco et al., 1993) (compare Fig. 5 *a* to 5 *c*, *small arrows*). In contrast, ~70–80% of the cells microinjected with the S25N mutant (Fig. 5, *d–f*) showed nearly complete retention of VSV-G in the ER as indicated by the diffuse

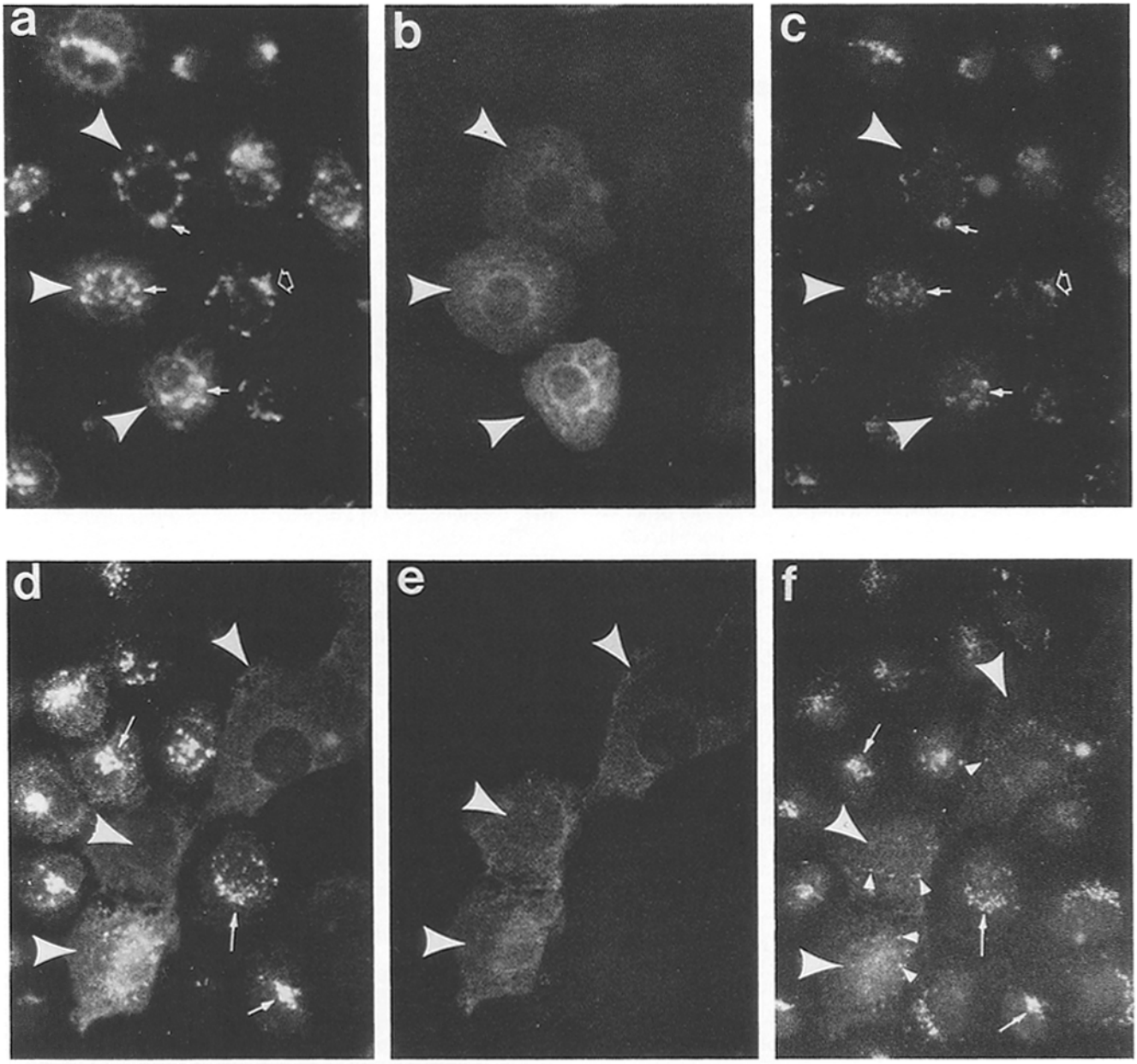


Figure 5. Microinjection of recombinant rabla(S25N) inhibits protein export from the ER and triggers dispersal of the Golgi complex. NRK cells were infected with the ts045 strain of VSV at the restrictive temperature and microinjected with a 10–20-fold excess of recombinant rabla (*a–c*) or rabla(S25N) (*d–f*) over the endogenous rabl pool in combination with an IgG marker as described in Materials and Methods. After a 1.5-h incubation at the restrictive temperature, the cells were shifted to the permissive temperature for 1.5 h, and the distribution of VSV-G (*a* and *d*), IgG (*b* and *e*), and Man II (*c* and *f*) were determined by indirect immunofluorescence as described in Materials and Methods. The large arrowheads denote the microinjected cells. The small arrows in panels *a* and *c* denote colocalization of VSV-G with representative Golgi structures in cells injected with wild-type rabla; the open arrow in panels *a* and *c* points to a Golgi structure in a non-injected cell. The arrows in panels *d* and *f* indicate Golgi in non-injected cells; the small arrowheads in panel *f* denote dispersed Golgi fragments in cells injected with the rabla(S25N) mutant.

staining pattern (Fig. 5 *d*), although in some cells variable degrees of transport to small punctate structures reminiscent of pre-Golgi intermediates (Plutner et al., 1992) could be detected (Fig. 5 *d*). Strikingly, injected cells which showed no or little export of VSV-G from the ER also lacked recognizable Golgi structures as detected by the distribution of Man II (Fig. 5 *f*, compare injected [*large arrowheads*] to non-injected cells). In some cases, structures containing Man II, but not VSV-G, could be detected throughout the cytoplasm (Fig. 5 *f*, *small arrowheads*). There was no apparent redistribution of Man II to the ER (Fig. 5 *f*), a result consistent with the nearly complete lack of processing of VSV-G to the R_1 form (Fig. 2). Thus, microinjection of the S25N mutant dramatically perturbed the structural integrity of the Golgi apparatus in a fashion which was distinct from that of brefeldin, A (BFA), a drug which promotes the collapse of the Golgi

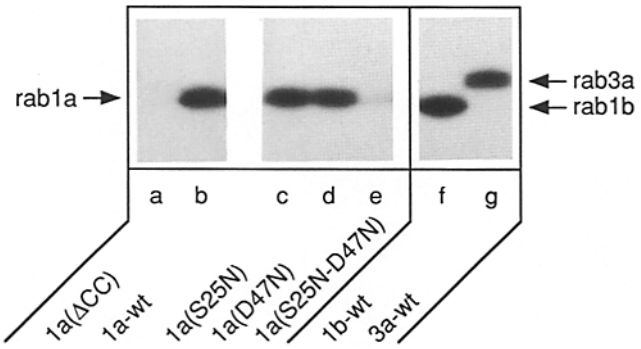


Figure 6. Recombinant rabla(S25N) can be posttranslationally prenylated in vitro. 1 μg (40 pmol) of the indicated recombinant wild-type and mutant proteins were incubated for 1 h at 32°C in the presence of rat liver cytosol, ATP, and 0.52 mM [^{3}H]GGPP (19.3 Ci/mmol) as described in Materials and Methods. The samples were analyzed by SDS-PAGE and fluorography.

stack into the ER (Lippincott-Schwartz, 1993) and processing of VSV-G (Doms et al., 1989), or from nocodazole, a microtubule-depolymerizing agent which disperses the central Golgi stack to numerous peripheral fragments (Turner and Tartakoff, 1989) (data not shown).

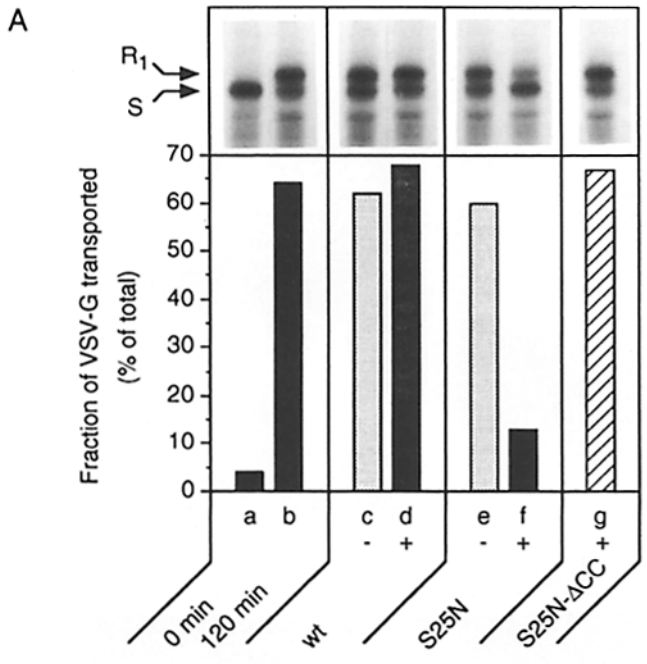
Rabla(S25N) Inhibits Export of VSV-G from the ER in Perforated Cells

The above results raised the possibility that the apparent inhibitory effect of the S25N mutant on ER to Golgi transport might be indirect—due to dismantling of the Golgi stack. A technical limitation inherent to the transient expression and microinjection approaches is the incubation time required for the accumulation of functional protein in vivo. To identify the immediate effect of the mutant on transport, we established conditions which support posttranslational processing of recombinant rabl wild-type and mutant proteins in vitro. For this purpose, recombinant rabl proteins were incubated with [^{3}H]GGPP in the presence of cytosol prepared from rat liver homogenates. While no labeling was detected using the rabla(ΔCC) mutant lacking the carboxyl-terminal Cys residues (Fig. 6, lane *a*), comparable levels of [^{3}H]GG were incorporated into both wild-type rabla and the S25N mutant (Fig. 6, lanes *b* and *c*). Similar results were obtained with the wild-type rablb and rab3a proteins (Fig. 6, lanes *f* and *g*). These results demonstrate that the recombinant proteins have intact carboxyl-terminal sequences and provide evidence that rat liver cytosol contains prenyl transferase(s) which support modification of both CC and the CXC motifs, the latter being found at the carboxyl-termini of rab3 and several other members of the rab family. In contrast to previous results with the equivalent rablb mutant (Wilson and Maltese, 1993), we found that the D47N mutation did not prevent the incorporation of [^{3}H]GG into rabla(D47N) (Fig. 6, lane *d*). However, the rabla(S25N-D47N) double-mutant could not be prenylated (Fig. 6, lane *e*). The latter result provides an unexpected, but plausible explanation for the ability of the D47N substitution to neutralize the inhibitory potential of the rabla mutation.

Having established conditions to generate posttranslationally processed recombinant wild-type and rabla mutant proteins, we examined the effects of these proteins on transport using an assay which efficiently reconstitutes both ER to Golgi and intra-Golgi transport using perforated cells (Beckers et al., 1987; Davidson and Balch, 1993). The recombinant proteins were first incubated for 1 h in the presence of rat liver cytosol and GGPP to promote isoprenylation before addition to the assay. The efficiency of the modification reaction in this case was estimated from the fraction of rabla protein recovered in the detergent phase after phase separation in Triton X-114 solution (Bordier, 1981) and was generally found to be ~5–10% of the total rabla added (data not shown). No processing or partitioning of rabl into the detergent phase occurred in the absence of GGPP (data not shown). Aliquots of the posttranslationally modified proteins were added to transport assays which contained ~5–10 ng of endogenous rabl. As shown in Fig. 7, wild-type rabla preincubated in the absence (~1 μg of unprocessed protein) or presence of GGPP (~70–90 ng of processed protein) neither stimulated nor inhibited ER to Golgi transport (Fig. 7 *A*, lanes *c* and *d*; *B*, *open circles*). In contrast, inhibition was achieved with the S25N mutant after preincubation in the presence of GGPP (Fig. 7 *A*, lane *f*). The amount of processed rabla(S25N) required for complete inhibition was ~80–100 ng with an IC_{50} in the range of ~40 ng (Fig. 7 *B*). This value represents a 5–10-fold excess over the endogenous pool. Omitting GGPP from the preincubation cocktail eliminated the inhibitory potential of the S25N mutant (Fig. 7 *A*, lane *e*). In addition, no inhibition was observed with the rabla(S25N-ΔCC) mutant lacking the carboxyl-terminal Cys residues (Fig. 7 *A*, lane *g*). Supplementing the assay with an equivalent amount of processed wild-type rabla or rablb, but not rab3a, efficiently reversed the inhibition (Fig. 8, lanes *c*, *e*, and *f*). Wild-type rabla failed to restore transport if preincubated in the absence of GGPP (Fig. 8, lane *b*). The combined results illustrate that the phenotype of the S25N mutant in vitro is identical to that observed in vivo and requires isoprenylation for function.

Rabla(S25N) Inhibits Export of Protein from the ER

To identify the stage at which the rabla(S25N) mutant inhibits transport, NRK cells were infected with tsO45 VSV at the restrictive temperature, transferred to ice, and permeabilized using conditions which allow us to follow the migration of tsO45 VSV-G from the ER to the Golgi in vitro using indirect immunofluorescence (Plutner et al., 1992). These cells were subsequently incubated for 30 min at 32°C with prenylated forms of wild-type rabla or the rabla(S25N) mutant at concentrations ~20-fold over the endogenous rabla pool to insure strong inhibition. As shown in Fig. 9, in the absence of recombinant protein tsO45 VSV-G redistributed from the ER (Fig. 9 *a*) to intensely labeled punctate structures (Fig. 9 *c*) consisting of preGolgi intermediates and Man II containing Golgi compartments (Fig. 9 *c* [VSV-G] and *d* [Man II]) (Plutner et al., 1992). A similar pattern was observed upon incubation with wild-type rabla (Fig. 9 *e* [VSV-G] and *f* [Man II]). In contrast, no overlap of VSV-G with the Golgi marker could be detected in the presence of rabla(S25N) (Fig. 9 *g* [VSV-G] and *h* [Man II]), a result which is consistent with the lack of processing to the endo H-resistant R_1 form (see Fig. 2). Instead, VSV-G largely remained in a diffuse ER staining pattern and could be detected, albeit infrequently, in weakly staining punctate structures (Fig. 9 *g* [*boxed region* and *arrowheads*]). These results



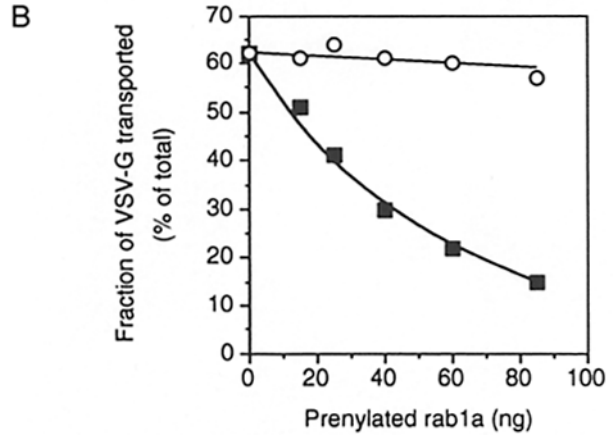


Figure 7. Recombinant rabla(S25N) inhibits ER to Golgi transport in vitro. NRK cells infected with the ts045 strain of VSV were pulse-labeled with Trans ^{35}S-label at the restrictive temperature, transferred to ice, and perforated as described in Materials and Methods. (*A*) Reconstitution of the inhibitory phenotype of the S25N mutant in vitro. Perforated cells were incubated in the presence of rat liver cytosol and ATP for 2 h on ice (lane *a*) or at 32°C (lanes *b–g*). The reactions contained no additions (lanes *a* and *b*) or were supplemented with wild-type rabla (lanes *c* and *d*), rabla(*S25N*) (lanes *e* and *f*), or rabla(*S25N-ΔCC*) (lane *g*) which were preincubated with rat liver cytosol and ATP in the absence (−; lanes *c* and *e*) or presence (+; lanes *d*, *f*, and *g*) of GGPP as described in Materials and Methods. Endo H-sensitive (*S*) and endo H-resistant (R_1) forms of VSV-G were resolved by SDS-PAGE (*upper panel*) and the fraction of processed VSV-G quantitated by scanning densitometry (*lower panel*) as described in Materials and Methods. (*B*) Dose-dependent inhibition of transport in the presence of processed rabla(*S25N*). The wild-type (○) and mutant (■) proteins were prenylated in vitro as outlined above, and aliquots of the preincubation reactions containing the indicated amounts of processed protein were included into the transport cocktails. The extent of posttranslational modification was estimated using phase separation in Triton X-114 solution as described in Materials and Methods.

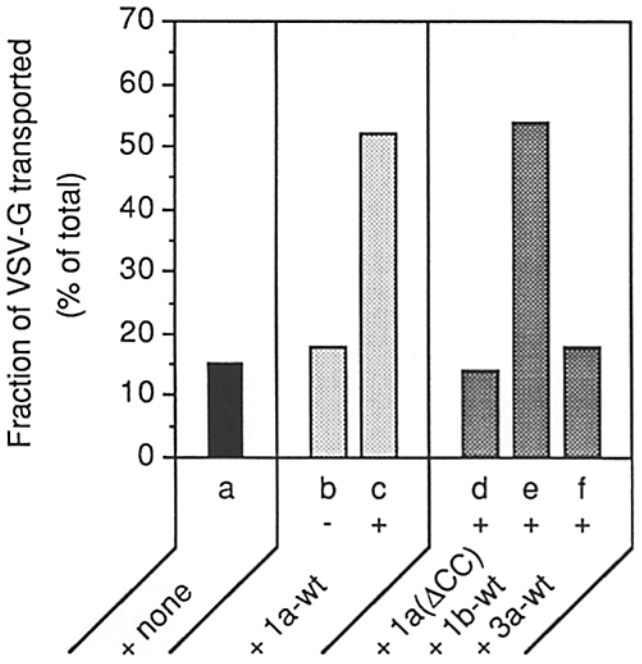


Figure 8. Wild-type rabla and rablb reverse rabla(S25N) mediated inhibition in vitro and require posttranslational prenylation for function. Perforated NRK cells were incubated in vitro with processed rabla(S25N) alone (lane *a*) and in combination with equivalent amounts of the indicated proteins (lanes *b–f*) preincubated in the absence (−; lane *b*) or presence (+; lanes *c–f*) of GGPP. Transport was assessed and quantitated as described in the legend to Fig. 8 and in Materials and Methods.

suggest that the S25N mutant leads to a marked reduction of export from the ER to preGolgi intermediates, similar to that observed when cells are incubated in the presence of limiting cytosol (Plutner et al., 1992; Peter et al., 1993). Importantly, throughout this relatively short incubation the Golgi compartments remained largely intact (Fig. 9, compare *d* and *h*). These results confirm that the primary consequence of the S25N mutant is to cause a reduction in the extent of vesicle budding from the ER. Under these conditions, vesicles escaping the ER are likely to contain the rabla-(S25N) mutant protein. This may also inhibit their targeting/fusion to the cis-Golgi compartment due to the inability of the mutant protein to adopt a conformation equivalent to the active GTP-bound state.

Discussion

In this study we have analyzed the role of the transition of rabl from the GDP-bound to the GTP-bound state in ER to Golgi traffic. The rabla(S25N) mutant, which shows a markedly reduced affinity for GTP, inhibits normal rabl function by blocking vesicle formation in a competitive fashion. These results provide the first direct evidence for the role of rabl at this step in transport, demonstrate that posttranslational isoprenylation is essential for rabl function, and indicate that the two isoforms of rabl, despite their divergent carboxyl-terminal domains, are functionally interchangeable. Moreover, the data show that the rabla(S25N) mutant also inhibits intra-Golgi transport and, unexpectedly,

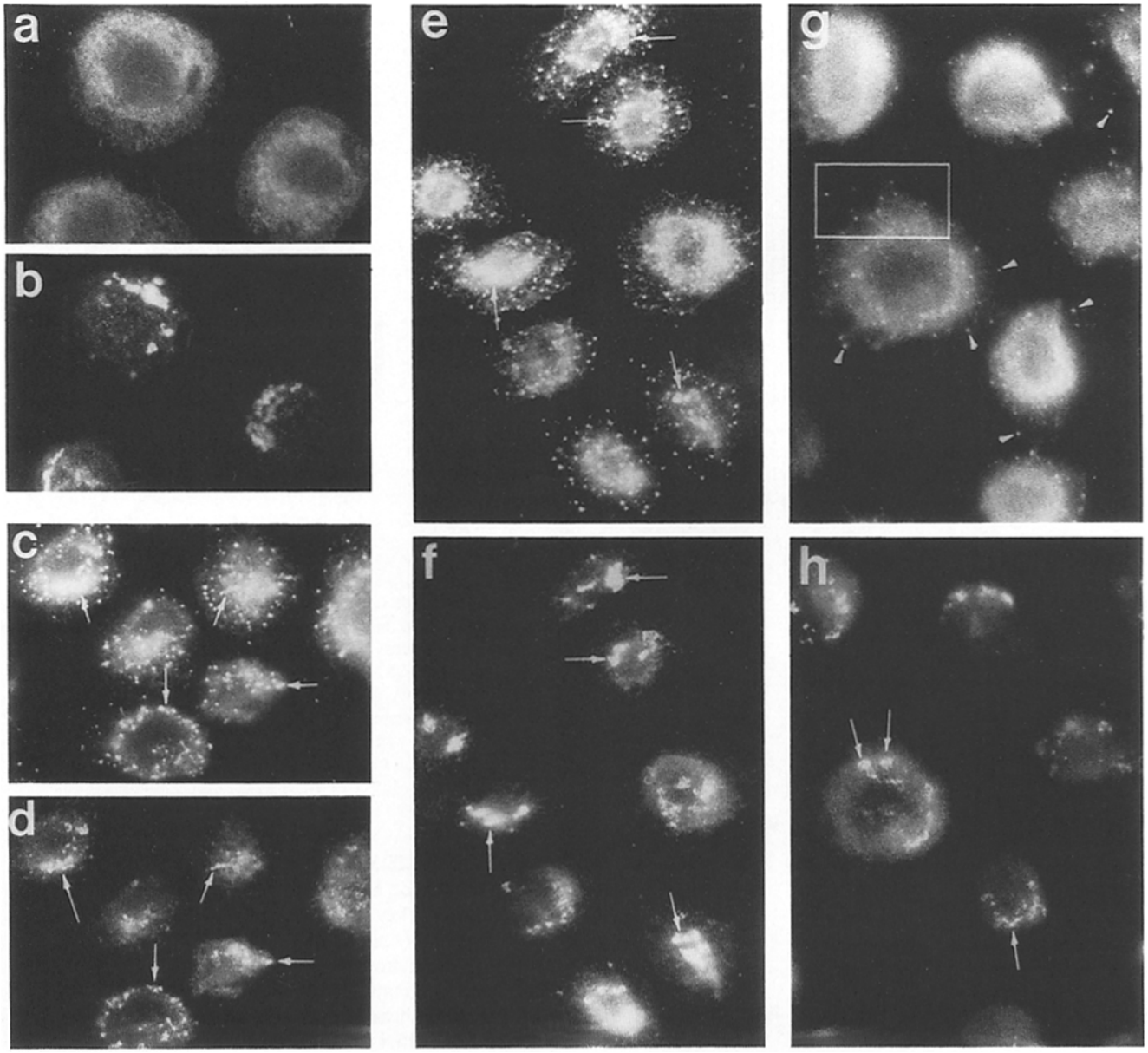


Figure 9. The rabla(S25N) mutant prevents export of VSV-G from the ER in vitro. NRK cells were infected with the ts045 strain of VSV at the restrictive temperature, permeabilized with digitonin, and incubated in the presence of cytosol and ATP for 30 min on ice (*a* and *b*) or at 32°C (*c–h*) as described in the Materials and Methods. The reactions contained no additions (*a–d*) or were supplemented with posttranslationally processed wild-type rabla (*e* and *f*) or rabla(S25N) (*g* and *h*). The distribution of VSV-G (*a*, *c*, *e*, and *g*) or Man II (*b*, *d*, *f*, and *h*) was determined by indirect immunofluorescence as described in Materials and Methods. The arrows in panels *c* and *d* or *e* and *f* denote colocalization of VSV-G with Man II. In the presence of the rabla(S25N) mutant VSV-G is largely retained in the ER (*g*) and is absent from the Golgi structures (*arrows* in *h*). The box and arrowheads in panel *g* illustrate markedly reduced formation of VSV-G containing preGolgi intermediates in the presence of the S25N mutant.

establish a close relationship between rabl function in vesicular traffic and the integrity of the Golgi apparatus.

Proper Mg^{2+} Coordination Is Critical for Rabl Function

Replacing the highly conserved Ser at position 25 of rabla with Asn converts the protein into a potent trans-dominant inhibitor of vesicular transport between the ER and the Golgi compartments both in vivo and in vitro. X-ray crystallographic studies have revealed that the hydroxyl group of the equivalent Ser-17 in H-ras contributes to the coordination of a Mg^{2+} ion, which binds to the β- and γ-phosphates of guanine nucleotides and is essential for GTP hydrolysis (Pai et al., 1989, 1990). Consistent with an important role of Mg^{2+} in stabilizing nucleotide binding, rabla required low concentrations of free Mg^{2+} for the rapid exchange of bound nucleotide for exogenous GDP or GTP in vitro. In contrast, the guanine nucleotide exchange capacity of the rabla(S25N) mutant was independent of the Mg^{2+} concentration, a result entirely compatible with the involvement of Ser-25 in Mg^{2+} coordination. Our results demonstrate that the S25N substitution decreases the affinity of the protein for GTP in vitro

by as much as two orders of magnitude, without affecting its affinity for GDP, although the mutant can still bind GTP in the presence of high nucleotide concentrations. It has been established that the equivalent H-ras(S17N) mutant remains unable to stimulate a downstream effector protein in vitro even upon binding of the non-hydrolyzable analog GTPγS (Farnsworth and Feig, 1991). Thus, it appears that the S17N substitution restricts the protein to an inactive, GDP-bound conformation. It has been proposed that this mutant inhibits growth by competing with the wild-type protein for a ras-GEP, critical for ras activation (Farnsworth and Feig, 1991). Given the biochemical properties common to the rabla-(S25N) and the analogous H-ras(S17N) mutant, a similar mechanism is likely to be responsible for the inhibitory activity of the rabla(S25N) mutant on transport between the ER and Golgi compartments.

Isoprenylation Is Essential for the Inhibitory Activity of the Rabla(S25N) Mutant and the Function of Wild-Type Rabl

The growth inhibitory phenotype of the H-ras(S17N) mutant can be overcome by coexpression of the mutant protein with normal or transforming forms of ras (Feig and Cooper, 1988). Similarly, we have demonstrated that wild-type rabla is able to antagonize the inhibitory potential of the rabla-(S25N) mutant both in vivo and in vitro, suggesting a competitive relationship between the mutant and the wild-type protein. Posttranslational isoprenylation of the carboxyl-terminal Cys residues is critical not only for the inhibitory activity of the S25N mutant, but also for the ability of the wild-type rabla to relieve the transport block. The results are consistent with the notion that isoprenylation is, in general, essential for the function of ras-like GTPases (Der and Cox, 1991; Newman and Magee, 1993). While the precise role of this modification remains unknown, one possibility, among others, is that prenyl groups are critical for interaction of rabl with GEP- and/or GDI-like regulators (Soldati et al., 1993; Ullrich et al., 1993). In any event, the competitive nature of the rabla(S25N) mutant allowed us for the first time to directly investigate the contribution of the highly homologous rabla and rablb proteins to the transport process. Our data support the view that these proteins, which share 92% sequence identity, represent functionally interchangeable isoforms, although we cannot exclude the possibility that they differ in a subtle fashion which may not be apparent in our present experimental systems. Isoforms are common to many rab proteins (Zerial and Stennark, 1993). In the case of rab3, however, recent evidence suggests that rab3b, but not rab3a, is critical for regulated secretion in rat anterior pituitary cells (Liedo et al., 1993). Thus, different isoforms may fulfill more specialized roles in different cell types.

Effector Domain Substitutions

To test for the role of the putative rabl effector domain in vesicular traffic, we examined the impact of selected substitutions (T43A and D47N) in this region on both wild-type and mutant rabla function. The T43A mutation was chosen because the equivalent substitution in H-ras has been shown to neutralize the transforming potential of various oncogenic forms (Haubruck and McCormick, 1991; Sigal et al., 1986), presumably by disrupting the interaction between the activated protein and a specific GTPase-activating protein (ras-GAP) (Adari et al., 1988; Calès et al., 1988). In addition, the analogous rab3a(T54A) mutant blocks interaction with a putative rab3-GEP in vitro (Burstein et al., 1992). The T43A substitution in rabl did not perturb activity, suggesting that this highly conserved Thr may be less critical for rabl function. The D47N substitution was based on evidence that the equivalent mutation in Yptlp (D44N) results in a temperature-sensitive phenotype, and more recently, that the D44N substitution in rablb prevents posttranslational isoprenylation in vitro (Wilson and Maltese, 1993). In contrast, we found that the rabla(D47N) mutant was efficiently prenylated and had functional activity equivalent to that of the wild-type protein in vivo and in vitro. An explanation for these apparently discrepant results remains to be established. However, the observation that the rabla(S25N-D47N) double-mutant was defective for prenylation provided an explanation for the ability of the D47N substitution to neutralize inhibition by the S25N mutant. Such effects will be important to consider when analyzing the capacity of other mutations to perturb rab wild-type and mutant function.

The S25N Mutant Inhibits Export from the ER and Affects Golgi Integrity

Morphologically, the presence of high concentrations of the S25N mutant led to a significant retention of VSV-G in the ER. This was particularly evident in vitro, but could also be detected upon microinjection of recombinant rabla(S25N) into intact NRK cells. We interpret these results to indicate that the S25N mutant markedly reduces the extent of vesicle budding from the ER. This could be due to perturbing the function of a rabl-specific GEP, a factor which is likely to be essential for rabl activation during recruitment of the protein before or coincident with vesicle formation. Carrier vesicles which do form even in the presence of excess rabla(S25N) mutant are likely to be fusion incompetent, given that the rabl mutant, like the equivalent ras mutant (Farnsworth and Feig, 1991), may be unable to undergo conformational changes normally associated with GTP-binding.

Inhibition of vesicular traffic by rabla(S25N) was accompanied by an apparent dispersal of the Golgi apparatus based on the distribution of Man II. Even at the level of indirect immunofluorescence we found this result to be distinct from the morphological effect of the microtubule-depolymerizing agent nocodazole (which leads to fragmentation of the Golgi to small stacks [Turner and Tartakoff, 1989]), and from that of BFA (for review see Klausner et al., 1992; Lippincott-Schwartz, 1993) or the Arf1(T31N) mutant containing a substitution equivalent to that in rabla(S25N) (Dascher and Balch, 1994). Both BFA and the Arf1(T31N) mutant promote release of β-COP from Golgi membranes (Dascher and Balch, 1994; Lippincott-Schwartz, 1993) and the retrograde delivery of resident Golgi proteins to the ER. In both cases, tsO45 VSV-G is processed to endo H-resistant forms (Doms et al., 1989; Dascher and Balch, 1994). In contrast, the rabla(S25N) mutant did not promote redistribution of Golgi processing enzymes to the ER, and VSV-G largely remained in the endo H-sensitive form. These results provide new evidence which link the structural organization of the Golgi complex to rabl and its associated components involved in vesicle traffic. The effect of the S25N mutant on the struc-

tural integrity of the Golgi stack has been characterized at high resolution elsewhere (Wilson et al., 1994).

It is now apparent from these studies that rabl has biochemical properties which are in part similar and, in part, different from those of H-ras. Identification of the upstream and downstream components required for rabl function should prove useful in understanding the molecular interactions responsible for these differences and in elucidating the different roles that rabl may have in vesicle budding, targeting, and fusion. In the accompanying manuscript (Pind et al., 1994), we address the role of a rabla mutant which has a high guanine nucleotide exchange rate (rabl[N124I]) in specifically inhibiting carrier vesicle targeting/fusion.

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