Sar1 Promotes Vesicle Budding from the Endoplasmic Reticulum but Not Golgi Compartments

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Abstract. Two new members (Sarla and Sarlb) of the SAR1 gene family have been identified in mammalian cells. Using immunoelectron microscopy, Sar1 was found to be restricted to the transitional region where the protein was enriched 20–40-fold in vesicular carriers mediating ER to Golgi traffic. Biochemical analysis revealed that Sar1 was essential for an early step in vesicle budding. A Sar1-specific antibody potently inhibited export of vesicular stomatitis virus glycoprotein (VSV-G) from the ER in vitro. Consistent with the role of guanine nucleotide exchange in Sar1 function, a *trans*-dominant mutant (Sarla[T39N]) with a preferential affinity for GDP also strongly inhibited vesicle budding from the ER. In contrast, Sar1 was not found to be required for the transport of VSV-G between sequential Golgi compartments, suggesting that components active in formation of vesicular carriers mediating ER to Golgi traffic may differ, at least in part, from those involved in intra-Golgi transport. The requirement for novel components at different stages of the secretory pathway may reflect the recently recognized differences in protein transport between the Golgi stacks as opposed to the selective sorting and concentration of protein during export from the ER.

MULTIPLE GTPases are now recognized to regulate vesicular traffic between compartments of the exocytic pathway. They include members of the Rab/YPT/SEC4, Sar1, Arf, and $G_{\alpha\beta\gamma}$ gene families (reviewed in Goud and McCaffrey, 1991; Barr et al., 1992; Bomsel and Mostov, 1992; Pfeffer, 1992; Zerial and Stenmark, 1993). These proteins are proposed to serve as molecular switches which monitor and coordinate sequential interactions between the components of transport machinery required for a single round of budding, targeting, and fusion of transport vesicles. Members of the Sar1 family are evolutionarily distant from both the Rab/YPT1/SEC4 family (<30% identity), but show slight homology to the Arf family. Only one member has been identified to date in *Saccharomyces cerevisiae* (Nakano and Muramatsu, 1989), although homologues have been found in *Schizosaccharomyces pombe* and *Arabidopsis thaliana* (d'Enfert et al., 1992). In yeast, Sarlp plays a key role in the export of protein from the ER (Nishikawa and Nakano, 1991; Oka et al., 1991; Salama et al., 1993). Sarlp function requires Sec12p (a Sarlp-specific guanine nucleotide exchange factor [GEF][1]) (Barlowe and Schekman, 1993) and Sec23p (a Sarlp-specific GTPase activating protein [GAP]) (Yoshihisa et al., 1993). Recently, these components in addition to three other soluble proteins have been purified to homogeneity and demonstrated to be sufficient for vesicle budding from the ER in yeast (Nakano et al., 1988; Hicke and Schekman, 1989; d'Enfert et al., 1991; Hicke et al., 1992; Barlowe et al., 1993; Pryer et al., 1993; Salama et al., 1993; Yoshihisa et al., 1993). In mammalian cells, Sar1 function has been indirectly linked to the requirement for β-COP in vesicle budding from the ER (Peter et al., 1993). The possible involvement of Sarlp in other steps of the secretory pathway has not been investigated.

To begin to understand the similarities and differences between export from the ER and vesicular traffic through the Golgi apparatus in mammalian cells, we have identified two closely related mammalian homologues of yeast Sarlp (designated Sarla and Sarlb). We find that Sar1, unlike either Arf1 (Orci et al., 1991*b*, 1993; Serafini et al., 1991; Kahn et al., 1992; Taylor et al., 1992; Palmer et al., 1993) or Rab1 (Davidson and Balch, 1993; Nuoffer et al., 1994; Pind et al.,

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1. *Abbreviations used in this paper*: endo H, endoglycosidase H; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; RER, rough endoplasmic reticulum; VSV-G, vesicular stomatitis virus glycoprotein; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate).

1994), is not required for vesicular traffic between Golgi compartments. These results provide novel evidence that small GTPases (and their associated effectors) regulating vesicle budding from the ER and from compartments of the Golgi may, in part, be distinct. This could reflect differences in the need for these two organelles to recruit cargo to vesicular carriers (Balch et al., 1994; Pind et al., 1994).

Materials and Methods

cDNA Cloning

Degenerate oligonucleotide mixtures corresponding to regions of identity among the reported SAR1 protein sequences of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Arabidopsis thaliana* were used to amplify Sar1 cDNA from a CHO cDNA library (Uni-ZAP XR; Stratagene, La Jolla, CA) by two stage PCR. The primers used for the first round of amplification were GG(ATGC)(TC)TIGA(TC)AA(TC)GC(AGTC)GG (codons Gly^{32}–Gly^{37}, sense) and (GC)(AT)(AG)CACAT(AG)AA(AGTC)-AC(TC)TC (codons Glu^{174}–Ser^{179}, antisense). The same antisense primer and GA(TC)AA(TC)GC(AGTC)GG(AGTC)AA(AG)AC (codons Asp^{34}–Thr^{40}, sense) were used for the second round of amplification. All nucleotides in parentheses were included at that position. The amplification reactions were performed for five cycles with a denaturing temperature of 94°C for 1 min, annealing at 47°C for 1 min, and elongation at 72°C for 2 min, followed by an additional 25 cycles with a denaturing temperature of 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. For the second round of amplification, the first round reaction was diluted 10,000-fold and used as the template. The 0.4-kb product of the second round amplification was subcloned into a plasmid, pBluescript II (Stratagene), sequenced, and used as a probe for screening a CHO cDNA library constructed with the bacteriophage λ vector (Uni-ZAP XR; Stratagene). Hybridizations were done for 24 h at 65°C in 5× SSPE (1× SSPE = 0.15 M NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.4), 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml herring sperm DNA. A final wash was performed in 1× SSPE, 0.1% SDS at 65°C for 1 h. Eleven positive λ clones were isolated, and the DNA inserts with the vector sequence carried by the λ clones were rescued by in vivo excision according to the manufacturer's procedure, analyzed by restriction enzyme digestion and partial sequencing, and then restricted to two sets of overlapping cDNAs. Both strands of each of the longest cDNAs termed Sar1a and Sar1b DNA, respectively, were fully sequenced with Sequence (United States Biochemical Corp., Cleveland, OH) using walking primers. Plasmids, pBluescript II carrying Sar1a and Sar1b cDNA, were termed pBluescript-Sar1a and pBluescript-Sar1b, respectively.

Expression of His_6-tagged Sar1a, Sar1b, and Sar1a-(T39N) Proteins in Escherichia coli

The coding region of Sar1a, Sar1b, and Sar1a(T39N) (see below) cDNAs were engineered by PCR to add a BamHI site and a sequence (ATCGAGGG-TAGA) corresponding to a factor Xa cleavage site immediately upstream of the first ATG and to add a HindIII site immediately downstream of the termination codon. The PCR products were digested with BamHI and HindIII and cloned into the pQE9 vector (QIAGEN Inc., Chatsworth, CA). The resulting plasmids, termed pQE-Sar1a, pQE-Sar1b, and pQE-SAR1a-T39N, respectively, were introduced into *Escherichia coli* (M15 harboring plasmid pREP4; QIAGEN). The transformants were grown to a density of A600 = 0.9 in Super medium (25 g bacto-tryptone, 15 g yeast extract, and 5 g NaCl per liter with 100 μg/ml ampicillin and 500 μg/ml kanamycin) at 37°C and further cultivated for 2 h in the presence of 2 mM isopropyl-thio-β-D-galactoside at 37°C to induce the recombinant proteins.

Purification of His_6-tagged Sar1 Proteins

Method 1. His_6-tagged Sar1a protein purified by this procedure was used as an antigen to produce polyclonal antibody. *E. coli* transformants expressing His_6-tagged Sar1a protein were suspended in sonication buffer (50 mM Na-phosphate [pH 8.0], 0.3 M NaCl) containing 1 mg/ml lysozyme, incubated on ice for 10 min, and then disrupted by sonication. The lysate was clarified by centrifugation at 10,000 *g* for 30 min, and the supernatant was loaded on a nickel-nitrotriacetic acid (Ni-NTA)–agarose (QIAGEN Inc.) column (8-ml bed volume) previously equilibrated with sonication buffer. The resin was washed with sonication buffer containing 20 mM imidazole and then His_6-tagged Sar1a protein was eluted with a 100-ml imidazole gradient (20–500 mM) in sonication buffer. His_6-tagged Sar1a protein was pooled and precipitated with 60% saturated ammonium sulfate, dissolved in PBS, and then dialyzed against PBS.

Method 2. His_6-tagged Sar1a proteins purified by this procedure were used for cell-free transport assays. *E. coli* transformants expressing His_6-tagged wild-type Sar1a and the T39N mutant were suspended in buffer A (10% glycerol, 150 mM KCl, 5 mM magnesium acetate, 0.1 mM DTT, 20 mM Hepes-KOH, pH 7.0) containing 1 mM PMSF, disrupted in a French press, and then the lysate was clarified by centrifugation at 10,000 *g* for 30 min. The supernatant was loaded on a Ni-NTA–agarose column (1-ml bed volume) previously equilibrated with buffer A and washed with buffer A containing 20 mM imidazole. The His_6-tagged protein was eluted with 7 ml of buffer A containing 100 mM imidazole, precipitated with 60% saturated ammonium sulfate, dissolved in buffer A, and dialyzed against buffer A.

Preparation of Antibody to Sar1a Protein

A rabbit was injected with 1 mg purified His_6-tagged Sar1a protein emulsified in Freund's complete adjuvant, followed with 1 mg purified His_6-tagged Sar1a protein emulsified in Freund's incomplete adjuvant every three weeks to boost the immune response. Antibody to Sar1a protein was affinity-purified as described (Harlow and Lane, 1988) by adsorption to His_6-tagged Sar1a protein conjugated to AminoLink® coupling gel (Pierce Chem. Co., Rockford, IL) according to the manufacturer's procedure, precipitated with 60% saturated ammonium sulfate, dissolved in 10 mM MOPS-KOH (pH 7.5) and 150 mM KCl, and dialyzed against the same buffer.

Construction of Sar1a Mutants

The T39N and N134I mutations were introduced into the wild-type Sar1a sequence carried by pBluescript-Sar1a, using site-directed mutagenesis as described (Higuchi et al., 1988). The resulting plasmids were termed pBluescript-Sar1a-T39N and pBluescript-Sar1a-N134I, respectively. For transient expression of wild-type and mutant Sar1a proteins in HeLa cells, the coding regions of wild-type and mutant Sar1a cDNAs were engineered by PCR to add a NdeI site immediately upstream of the first ATG and a BamHI site immediately downstream of the termination codon, using pBluescript-Sar1a, pBluescript-Sar1a-T39N, and pBluescript-Sar1a-N134I as templates. The PCR products were digested with NdeI and BamHI, and subcloned into these restriction enzyme sites of the pET3a vector (Novagen, Inc., Madison, WI) for expression from the T7 promoter. All mutant and wild-type Sar1 sequences engineered by PCR were verified by DNA sequencing.

Immunoblot Analysis

Proteins were fractionated by 12.5% polyacrylamide SDS-PAGE under reducing conditions (Laemmli, 1970) and electroblotted on to nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol at 22 V/cm for 1 h. Sar1 proteins were detected using the affinity-purified anti-Sar1a antibody (25 ng/ml) and peroxidase-conjugated anti-rabbit IgG (diluted 1:1,000). Peroxidase labeling was detected by chemiluminescence using the ECL reagent (Amersham Corp., Arlington Heights, IL) according to the manufacturer's recommendations.

Cis- to Medial-Golgi Transport Assay

The preparation of assay components and standard incubation conditions were as described previously (Balch et al., 1984; Malhotra et al., 1988). Reactions (50 μl) contained 25 mM Hepes (pH 7.0), 25 mM KCl, 2.5 mM magnesium acetate, 5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, 8 IU/ml creatine kinase, 8 μM palmitoyl CoA, 0.4 μM UDP-^{3}H-*N*-acetylglucosamine ([^{3}H]GlcNac), bovine brain cytosol (5 μl), donor stacks (5 μl), and acceptor stacks (5 μl). To test the effect of anti-Sar1a antibody, the reactions were incubated on ice for 1 h to promote antibody-antigen binding and then incubated at 37°C for assay.

Transport in Semi-intact and Permeabilized Cells

NRK cells were infected with the ts045 strain of vesicular stomatitus virus (VSV) and pulse labeled with 10 μCi Trans [^{35}S]-label at the restrictive temperature (39.5°C) to accumulate the VSV-glycoprotein (VSV-G) mutant in the ER. The cells were then perforated by the swelling and scraping pro-

cedure as described (Balch et al., 1986; Beckers et al., 1987; Davidson and Balch, 1993). Transport between the ER and the *cis/medial*-Golgi compartments was measured biochemically by following the appearance of endoglycosidase H (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 (Plutner et al., 1992; Davidson and Balch, 1993). 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 creatine phosphate, and 0.2 IU rabbit muscle creatine phosphokinase, final concentrations), 2–6 μl rat liver cytosol (20–60 μg/ml protein in 35 mM Hepes-KOH [pH 7.2], 125 mM KOAc), and 5 μl semi-intact cells (25–30 μg protein or 1–2 × 10^5 cells in 50 mM Hepes-KOH [pH 7.2], 90 mM KOAc). 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 (Balch et al., 1986; Plutner et al., 1992; Davidson and Balch, 1993). Autoradiographs were quantitated by a PhosphorImager (Molecular Dynamics, Palo Alto, CA).

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 (Balch, 1990; Plutner et al., 1992). An antibody specific for ManII (Velasco et al., 1993) was generously provided by M.G. Farquhar (Division of Cellular and Molecular Medicine, University of California, San Diego, CA).

Transient Expression and Analysis of Transport in HeLa Cells

Experimental procedures for the transient expression of pET-Sar1 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-Sar1 constructs using the Transfect ACE™ (GIBCO BRL, Gaithersburg, MD) procedure. After pulse labeling the cells for 10 min with 20 μCi Trans [^{35}S]-label (1,192 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 for endo H-sensitive to endo H-resistant forms as described (Tisdale et al., 1992; Davidson and Balch, 1993). 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-Sar1 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).

Immunocytochemistry

Indirect Immunofluorescence. Monolayer cultures of NRK cells, CHO-cells, and islet cells of neonatal rat pancreas were fixed with Bouin's fluid. Cells were permeabilized by dehydration and rehydration with ethanol and processed by the immunofluorescence technique. Affinity-purified rabbit antibodies raised against bacterially expressed His_6-tagged Sar1a protein (40–80 μg IgG/ml) were applied for 2 h at room temperature in a moist chamber followed by washing with PBS and exposure to FITC-conjugated goat anti-rabbit IgG. Cells were washed with PBS and counterstained with 0.03% Evans blue before examination with a confocal fluorescence microscope. As a control, preimmune IgG was applied instead of the primary antibody.

Immunoelectronmicroscopy. Fragments of pancreatic tissue freshly removed from normal adult rats were fixed with 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.4). After 1 h of fixation, the tissue was washed with buffer, infiltrated with sucrose, and processed for cryoultramicrotomy as described by Tokuyasu (1980). Islets of Langerhans isolated from rat pancreas by collagenase digestion were similarly fixed and processed. Immunolocalization of Sar1 on cryosections was carried out by the protein A-gold technique. Thin sections were incubated at room temperature with affinity-purified anti-Sar1 antibodies. Sections were subsequently washed with PBS, exposed to the protein A-gold solution (gold particles size 10 nm), and absorption stained with uranyl acetate according to Tokuyasu (1986).

Quantitative Evaluation. For quantitation of the immunolabeling, fields of insulin cells showing Golgi regions and associated transitional areas were photographed and printed at a calibrated magnification of 78,864×. Transitional areas were defined as the areas of the cytoplasm bordered by transitional elements of the ER and cisternae of the Golgi apparatus, and containing the transfer vesicles. For the quantitative evaluation of Sar1 immunolabeling, the transitional areas or the vesicles were delimited with an electronic pen and the number of gold particles in this area was recorded with the same pen connected to a microprocessor programmed to calculate the number of gold particles per unit area (μm^2). Gold particles were also quantitated on RER and Golgi stacks. On the latter, a line was traced in the middle of the stack: the half proximal to the transitional area was quantitated as the *cis*-Golgi, the half distal to the transitional area as *trans*-Golgi.

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                1                                                          60
CHO (sar1a)     MSFIFDWIYS GFSSVLQFLG LYKKTGKLVF LGLDNAGKTT LLHMLKDDRL GQHVPTLHPT
CHO (sar1b)     *********E **N******* ****S***** ********** ********** **********
S. cerevisiae     MAGWDIFG WFRDVLASLG LWNKHGKLLF LGLDNAGKTT LLHMLKNDRL ATLQPTWHPT
S. pombe             MFIIN WFYDALAMLG LVNKHAKMLF LGLDNAGKTT LLHMLKNDRL AVMQPTLHPT
A. thaliana          MFLFD WFYGILASLG LWQKEAKILF LGLDNAGKTT LLHMLKDERL VQHQPTQHPT
Consensus                     F   L  LG L  K  K  F LGLDNAGKTT LLHMLK  RL     PT HPT

               61                                                          120
CHO (sar1a)     SEELTIAGMT FTTFDLGGHI QARRVWKNYL PAINGIVFLV DCADHERLLE SKEELDSLMT
CHO (sar1b)     ********** *********E ********** ********** *****S**M* **V**NA***
S. cerevisiae   SEELAIGNIK FTTFDLGGHI QARRLWKDYF PEVNGIVFLV DAADPERFDE ARVELDALFN
S. pombe        SEELAIGNVR FTTFDLGGHQ QARRLWRDYF PEVNGIVYLV DCCDFERLSE SKAELDALLA
A. thaliana     SEELSIGKIK FKAFDLGGHQ IARRVWKDYY AKVDAVVYLV DAYDKERFAE SKRELDALLS
Consensus       SEEL       F  FDLGGH   ARR W  Y        V LV D  D  R  E    EL  L

               121                                                         180
CHO (sar1a)     DETIANVPIL ILGNKIDRPE AISEERLREM FGLYGQTTGK GSVSLKELNA RPLEVFMCSV
CHO (sar1b)     ****S***** ********TD *****K***I ********** *N******** *M********
S. cerevisiae   IAELKDVPFV ILGNKIDAPN AVSEAELRSA LGLLN.TTG. ...SQRIEGQ RPVEVFMCSV
S. pombe        MEELARVPFL ILGNKIDAPG AISEDELKAA LGLYQ.TTGK G.VSKPVPGI RPIEVFMCSV
A. thaliana     DEALATVPFL ILGNKIDIPY AASEDELRYH LGLTNFTTGK GKVTLGDSGV RPLEVFMCSI
Consensus            VP  ILGNKID    A SE  L     GL   TTG    V        R  EVFMCS

               181
CHO (sar1a)     LKRQGYGEGF RWMAQYID
CHO (sar1b)     ********** **LS****
S. cerevisiae   VMRNGYLEAF QWLSQYI
S. pombe        VLRQGYGEGF KWLAQYV
A. thaliana     VRKMGYGEGF KWLSQYIN
Consensus           GY E F  W  QY
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Figure 1. Comparison of protein sequences of the members of the SAR1 family (single letter amino acid code). The ORFs of the corresponding cDNA clones were translated and compared by BESTFIT alignment (Devereux et al., 1984). The underlined areas indicate conserved GTP-binding domains found in all members of the ras-superfamily (Bourne et al., 1991; Wittinghofer and Pai, 1991). Asterisks denote identity between the CHO Sar1a and Sar1b sequences. Consensus (identical) amino acids found in all of the members of the SAR1 family are shown in the bottom line.

Results

Isolation of Mammalian Homologues of Yeast SAR1

Degenerate oligonucleotide mixtures corresponding to regions of homology between the reported *S. cerevisiae*, *S. pombe*, and *A. thaliana* sequences (d'Enfert et al., 1992) were used to generate DNA fragments from a cDNA library of CHO cells by the polymerase chain reaction. A cDNA fragment that encoded a SAR1-like protein was used as a probe to screen a CHO cDNA library. Two different cDNA clones were obtained, both of which encode SAR1-like proteins, designated Sar1a and Sar1b (Fig. 1). The mammalian Sar1a and Sar1b cDNAs contain open reading frames of 597 nucleotides, encoding proteins of 198 amino acids with predicted molecular masses of 22,413 and 22,388 D, respectively. Fig. 1 shows a comparison of the amino acid sequence of five Sar1 proteins cloned to date. Sar1a and Sar1b are 91% identical (Table I). They share 60–67% identity with yeast and plant Sar1p (Table I), indicating that the Sar1 family is evolutionarily conserved. In contrast, the Sar1 proteins are <30% identical to other members of the Ras superfamily (Table I), suggesting that they play a distinct role in vesicular trafficking. The regions which confer homology to other members of the Ras superfamily are the amino acids underlined in Fig. 1. These comprise the three highly conserved motifs involved in GTP binding and hydrolysis found in all GTP-binding proteins examined to date (Valencia et al., 1991; Wittinghofer and Pai, 1991).

Mammalian Sar1 Is Highly Enriched on Vesicular Carriers Found in the Transitional Region of the ER

To establish that the cDNAs isolated encoded functional Sar1 proteins, Sar1a and Sar1b cDNAs were cloned into the pQE9 vector to produce proteins containing histidine residues at their NH_2 termini (Hochuli et al., 1988). These proteins were expressed in *E. coli* and purified by affinity chromatography on nickel-nitrolotriacetic acid (Ni-TCA)-agarose (Fig. 2, *A* and *B*). Affinity-purified anti-Sar1a antibody was prepared and its specificity examined by Western blotting. The polyclonal antibody recognized two prominent proteins in CHO lysates with molecular masses of 27 to 28 kD (Fig. 3).

Table I. Percentages of Amino Acid Identities between SAR1, Human ARF, and Human RAB Proteins

	CHOa	CHOb	S.c.	S.p.	A.t.	ARF1	ARF5	rab1	rab2	rab6
CHOa	100	91	61	67	61	30	30	22	22	23
CHOb		100	61	65	60	29	30	22	22	25
S.c.			100	72	64	37	34	24	17	22
S.p.				100	67	35	35	26	19	21
A.t.					100	34	34	24	20	20
ARF1						100	80	25	24	19
ARF5							100	28	21	19
rab1								100	48	37
rab2									100	39
rab6										100

Identities of each pairs of proteins were obtained by BESTFIT program. Abbreviations used are *CHOA*, sar1a protein of CHO; *CHOB*, sar1b protein of CHO; *S.c.*, Sar1 protein of *S. cerevisiae*; *S.p.*, Sar1 protein of *S. pombe*; *A.t.*, Sar1 protein of *A. thaliana*.

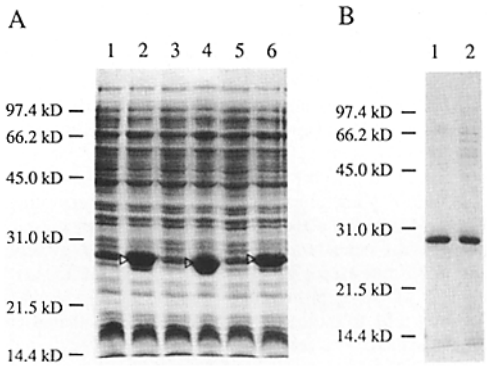

Figure 2. Expression and purification of wild-type and mutant proteins. (*A*) *E. coli* transformants harboring pQE-Sar1a (lanes *1* and *2*), pQE-Sar1b (lanes *3* and *4*), and pQE-Sar1a-T39N (lanes *5* and *6*) were grown to a density of A600 = 0.9 and further cultivated for 2 h at 37°C in the absence (lanes *1*, *3*, and *5*) or in the presence (lanes *2*, *4*, and *6*) of 2 mM isopropyl-thio-β-D-galactoside. Proteins of the cell lysates (30 μl culture) were fractionated by 12.5% polyacrylamide SDS-PAGE and stained with Coomassie blue. Open triangles indicate the induced Sar1 proteins. (*B*) Wild-type Sar1a (lane *1*) and Sar1a(T39N) mutant (lane *2*) proteins purified by the method 2 as described in Materials and Methods were run on 12.5% polyacrylamide SDS-PAGE (Laemmli, 1970) and stained with Coomassie blue.

The mobilities of these two species correspond to the slightly differing mobilities of recombinant His-tagged Sar1a and Sar1b (Fig. 3), suggesting that these two species may be the endogenous proteins corresponding to the cDNA clones.

The affinity-purified anti-Sar1a antibody was used to determine the distribution of Sar1 in mammalian cells. Using indirect immunofluorescence, Sar1 was not routinely abundant in the extensive cisternae comprising the bulk of the ER membrane. Rather, Sar1 was largely localized to the juxtanuclear Golgi region in NRK (Fig. 4 *B*) and CHO cells (Fig. 4 *C*). In pancreatic insulin cells (Fig. 4 *A*), the Sar1a distribution had a more extended reticular pattern due to the highly amplified secretory compartments found in these cells. In general, the pattern of Sar1 distribution is in part characteristic of the distribution of p53 and p58, markers for pre-Golgi intermediates which are abundant in the juxtanuclear Golgi region (Schleifer et al., 1982; Schweizer et al., 1988, 1990, 1991; Saraste and Svensson, 1991).

Using immunoelectron microscopy, Sar1 immunogold particles were found in rat pancreatic insulin cells to be abun-

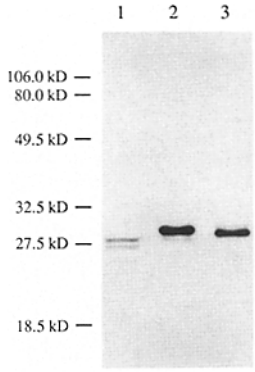

Figure 3. CHO cells contain two Sar1 isoforms. 60 μg protein of CHO cell lysate (lane *1*) and lysates (5 ng protein) of *E. coli* transformants expressing His_6-tagged Sar1a (lane *2*) and His_6-tagged Sar1b (lane *3*) were separated by 12.5% polyacrylamide SDS-PAGE and probed on Western blot with the affinity-purified anti-Sar1a antibody as described in Materials and Methods.

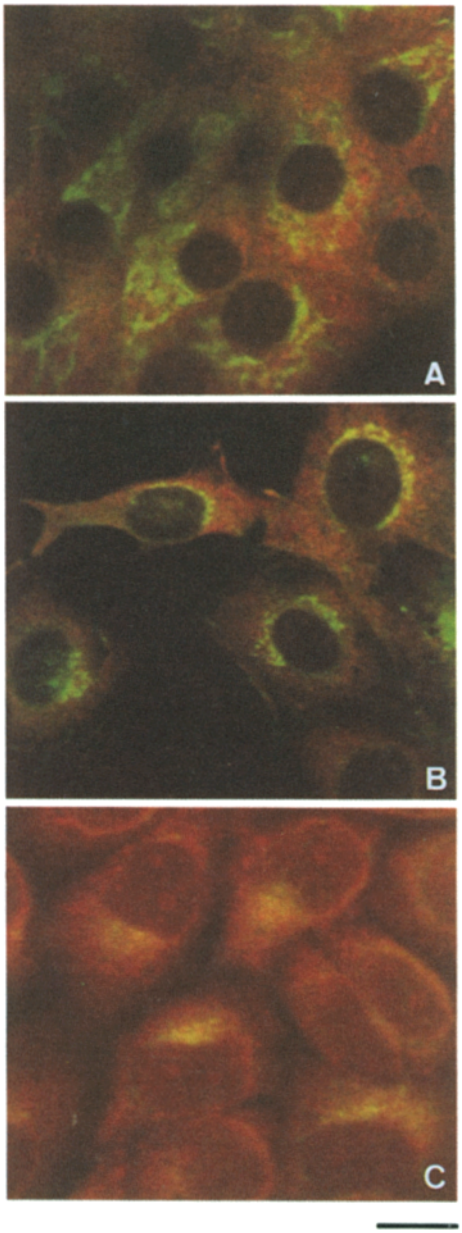

Figure 4. Sar1 is enriched in a juxtanuclear region. Immunofluorescence microscopy of insulin-secreting pancreatic endocrine cells (*A*), NRK cells (*B*) and CHO cells (*C*) stained with the affinity-purified anti-Sar1 IgG. A juxtanuclear fluorescent pattern corresponding to the location of the Golgi region is seen in the three cell types. In insulin cells, it has an extended reticular pattern due to the highly amplified secretory compartment. Bar, 20 μm.

dant on smooth transitional elements of the ER and on vesicular profiles in the proximal Golgi cisternae facing the transitional region (Fig. 5, *A* and *B*). The transitional region contained clusters of immunolabeled 40–60-nm vesicles, together with clusters of unlabeled vesicles. Sar1 was principally detected as a membrane-associated form (Table II). Quantitation of the distribution of Sar1 revealed that the carrier vesicles present in the transitional region were enriched approximately fourfold compared to the *cis*-Golgi cisternae and nearly 20-fold enriched compared to either the bulk of the ER or the *trans*-Golgi cisternae (Table II). The density on the *trans*-Golgi face was similar to that found over the nucleus (Table II), while the density over the bulk of the ER was approximately twofold greater than this level. Assuming the concentration of immunogold particles detected over the nucleus and the *trans*-Golgi compartments represents nonspecific background, then the enrichment of Sar1 in the pre-Golgi vesicular carriers is exceptionally high (up to 40–50-fold). These results are, in part, consistent with indirect immunofluorescence studies in yeast where Sar1p was detected in a diffuse, perinuclear, and reticular localization which overlapped with Kar2p and Sec62p, both resident ER proteins (Nishikawa and Nakano, 1991). Interestingly, Sar1p could also be detected in punctate structures in yeast which did not overlap with either ER or Golgi markers (Nishikawa and Nakano, 1991). These may represent pre-Golgi intermediates.

Sar1a(T39N) Inhibits Export of VSV-G from the ER In Vivo

To identify the potential role of Sar1a in the regulation of ER to Golgi traffic in mammalian cells, mutations were generated with motifs involved in guanine nucleotide interactions. Extensive mutational and structural analysis of Ras and Rab proteins have defined the essential amino acid residues in these motifs which are involved in the binding and hydrolysis of GTP (reviewed in Barbacid, 1987; Bourne et al., 1991). Two mutations were initially generated. These mutations are located in two of the four conserved guanine nucleotide-binding regions (Table III). The first mutant, Sar1a(N134I) contains a single point mutation (N134I) in the NKxD motif (residues 116–119 in $p21^{H\text{-}ras}$) which is essential for stabilization of the nucleotide-binding pocket (Pai et al., 1989, 1990). The equivalent substitution in $p21^{H\text{-}ras}$ (N116I) has a dominant negative phenotype and triggers oncogenic transformation. This mutant protein is defective in guanine nucleotide binding and has an exceptionally high exchange rate (Walter et al., 1986). In yeast, the corresponding mutations in the ypt1 or sec4 alleles (involved in ER to Golgi and post-Golgi transport, respectively) result in dominant lethal phenotypes which correlate with severe secretory defects (Schmitt et al., 1986, 1988; Walworth et al., 1989). In mammalian cells, an equivalent Rab1b(N121I) mutant inhibits ER to Golgi transport resulting in the accumulation of VSV-G in pre-Golgi intermediates in vivo (Tisdale et al., 1992) and in vitro (Pind et al., 1994).

The second mutant, Sar1a(T39N) carries a substitution in the GxxxxGKS/T domain (residues 10–17 in $p21^{H\text{-}ras}$) which

*Table II. Sar1 Immunogold Labeling of Transitional Area, ER and Golgi of Pancreatic β-cells**

	Number of gold particles per μm^2 ± SEM	
Nucleus	8 ± 3	‡n = 8
ER	19 ± 5	n = 8
Transitional area§	123 ± 23	n = 7
Transitional vesicles of transitional area	388 ± 72	n = 7
Cis Golgi	108 ± 23	n = 8
Trans Golgi	13 ± 3	n = 8

* Quantitation of the immunogold-labeled sar1 on compartments was performed as described in Materials and Methods.
‡ n = number of pictures evaluated.
§ 98 ± 2% of the particles was associated with the vesicles.

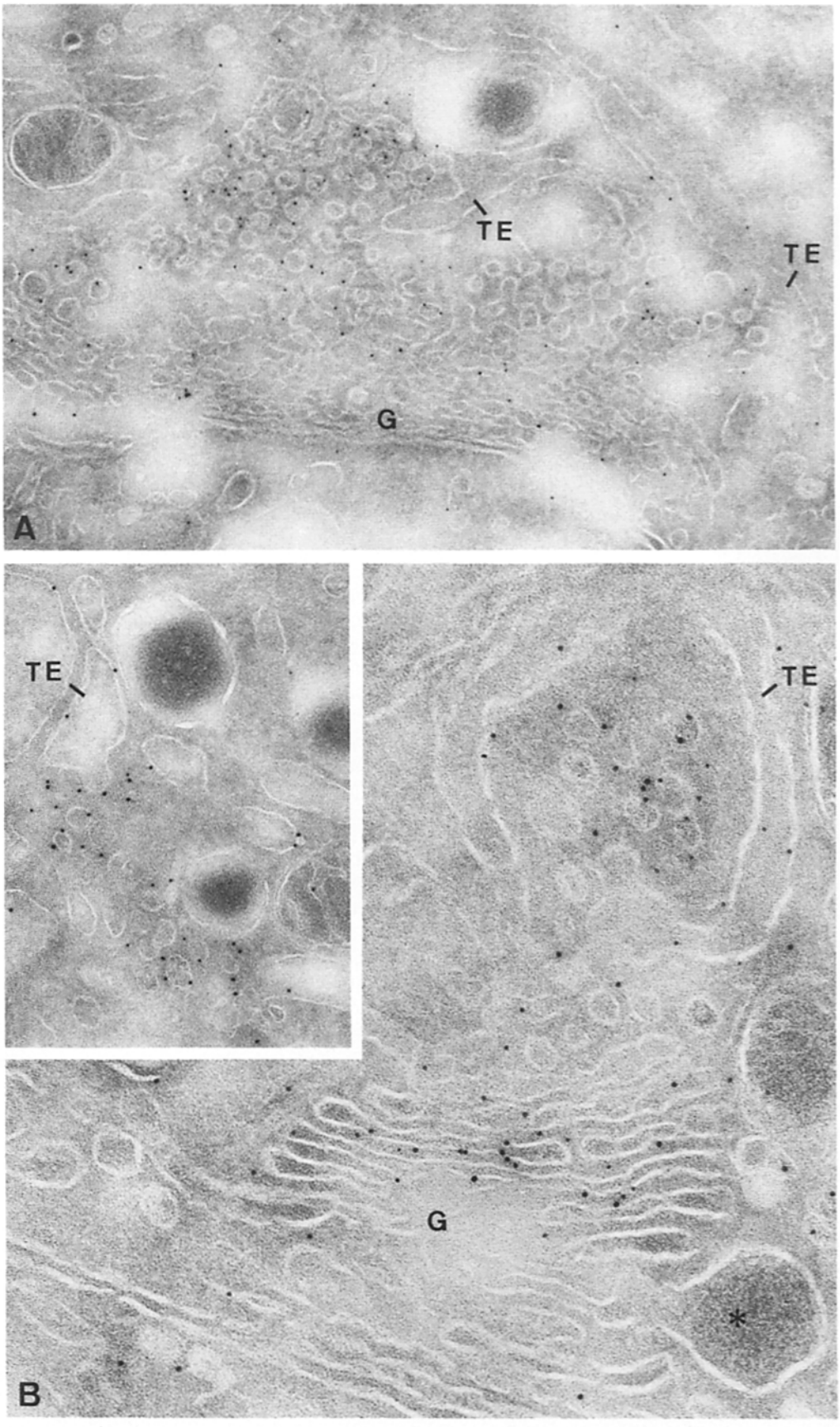

Figure 5. Immunogold distribution of Sarl in pancreatic β-cells. (*A*) Field of a pancreatic insulin cell comprising transitional elements (*TE*) of the ER, the proximal part of the Golgi complex (*G*) and abundant intervening vesicles. Sarl immunogold particles are preferentially associated with transitional elements and proximal Golgi cisternae. The transitional region contains clusters of immunolabeled vesicles, together with clusters of unlabeled vesicles. (*B*) Pancreatic insulin cell showing a bell-shaped transitional cisterna (*TE*), transfer vesicles and the Golgi complex (*G*). Sarl immunolabeling is associated with the transitional cisterna and vesicles, and the proximal Golgi cisternae facing the transitional region. The *trans*-Golgi cisterna is identified by a condensing insulin secretory vesicle (asterisk). It has a low level of Sarl labeling. The inset details Sarl-immunolabeled transitional elements (*TE*) and transfer vesicles. See the quantitative evaluation of immunolabeling in Table II. Bars: (*A* and *B*) 0.25 μm; (*B inset*) 0.21 μm.

Table III. Comparison of GTP-binding Domains of Sar1 to Other Members of the ras Superfamily

	5	53	112
H-ras (human)	NH2----KLVVVGAGGVGKS-----LDILDTAGQE-----VLVGNKCD----COOH		
rab1 (human)	NH2----KLLLIGDSGVGKS-----IQIWDTAGQE-----LLVGNKCD----COOH		
ARF1 (human)	NH2----RILMVGLGAAGKT-----FTVWDVGGQD-----LVFANKQD----COOH		
SAR1 (*S. cerevisiae*)	NH2----KLLFLGLDNAGKT-----FTTFDLGGHI-----VILGNKID----COOH		
SAR1 (*S. pombe*)	NH2----KMLFLGLDNAGKT-----FTTFDLGGHQ-----LILGNKID----COOH		
SAR1 (*A. thaliana*)	NH2----KILFLGLDNAGKT-----FKAFDLGGHQ-----LILGNKID----COOH		
Sar1b (Chinese hamster)	NH2----KLVFLGLDNAGKT-----FTTFDLGGHE-----LILGNKID----COOH		
Sar1a (Chinese hamster)	NH2----KLVFLGLDNAGKT-----FTTFDLGGHI-----LILGNKID----COOH		
		*	*
		Phosphoryl-binding site	Phosphoryl-binding site	Guanine ring contact site

The amino acid sequences of sar1-related proteins, which make up GTP-binding domain, are compared to H-ras and other members of the ras superfamily. The numbering is that of H-ras. Mutations used in this study which alter GTP-binding for ras are indicated by the asterisk.

is involved in phosphate binding and Mg^{2+} coordination (Pai et al., 1989, 1990). It is analogous, in principle, to the $p21^{H\text{-}ras}$(S17N) mutant, which is restricted to the inactive (GDP-bound) conformation and inhibits cell proliferation (Feig and Cooper, 1988; Farnsworth et al., 1991). The equivalent mutation in Rablb (Rablb[S22N]), is a potent inhibitor of both ER to Golgi and intra-Golgi transport in vivo (Tisdale et al., 1992) and in vitro (Davidson and Balch, 1993; Nuoffer et al., 1994). Competition experiments were performed to compare the relative affinities of the wild-type Sarla and the Sarla(T39N) mutant for GDP and GTP. The recombinant proteins were incubated with [^{3}H]-GDP (1 μM) in the presence of Mg^{2+} (1 mM) and increasing concentrations of unlabeled GDP (Fig. 6, *open symbols*) or GTP (Fig. 6, *closed symbols*). As expected, in the case of both the wild-type protein or mutant proteins, supplementing the reaction with ~1 μM GDP was sufficient to reduce [^{3}H]-GDP binding by ~50% (Fig. 6, *open symbols*). However, an excess of GTP was necessary for half-maximal inhibition of [^{3}H]-GDP binding for the wild-type and mutant proteins (Fig. *closed symbols*), suggesting that both proteins have a higher affinity for GDP than GTP. Moreover, an ~10-fold higher concentration of GTP was required for half-maximal inhibition of [^{3}H]-GDP binding to the Sarla(T39N) mutant that wild-type Sarla (Fig. 6, *closed symbols*), indicating that the predominant consequence of the T39N substitution with respect to guanine nucleotide binding is to reduce the affinity of the protein for GTP without altering its affinity for GDP.

To test if mutated forms of Sar1 inhibit ER to the Golgi transport in intact mammalian cells, a transient expression system was employed in which HeLa cells infected with vaccinia recombinant virus (vTF7-3) expressing T7 polymerase were cotransfected with a wild-type or mutant Sarla plasmid DNA and a plasmid encoding for VSV-G under control of the

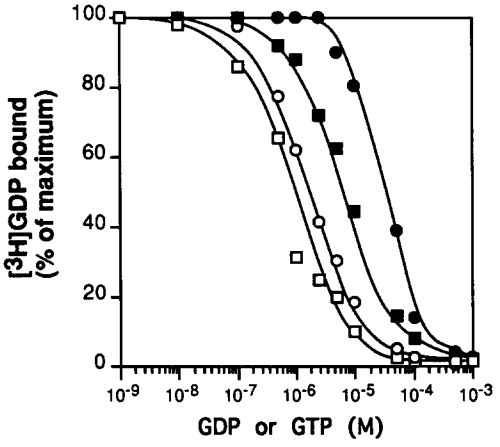

Figure 6. The Sarla(T39N) mutant has a reduced affinity for GTP. The wild-type (□, ■) and mutant (○, ●) proteins were incubated for 1 h with [^{3}H]-GDP in the presence of 0.1% Triton X-100 and 1 mM $MgCl_2$ and the indicated concentrations of unlabeled GDP (○, □) or GTP (●, ■). Exchange reactions were performed by incubating 0.5 μg (20 pmol) of recombinant protein at 32°C with 1 μM [^{3}H]-GDP (~5,000 cpm/pmol), 50 mM Hepes-KOH (pH 8.0), 0.1% Triton X-100, 1 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/ml BSA. Bound [^{3}H]-GDP was quantitated by liquid scintillation counting following capture of the proteins on nitrocellulose membranes as described previously (Nuoffer et al., 1994). The results are expressed as the percentage of the amount of [^{3}H]-GDP bound in the absence of additional nucleotide.

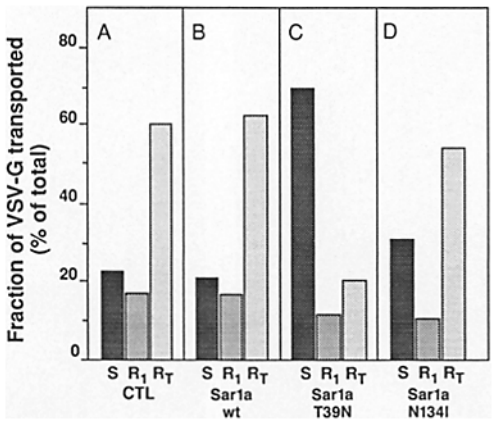

Figure 7. Sarla(T39N) ("GDP") potently inhibits ER to Golgi transport in vivo. Transient expression of wild-type VSV-G in combination with Sarla wild-type (*B*) or mutant proteins (*C* and *D*) in vaccinia-infected Hela cells was performed as described (Tisdale et al., 1992). The amount of VSV-G remaining in the endo H-sensitive ER form (*S*), the amount processed to the *cis/medial*-Golgi intermediate endo H-resistant forms (R_I) or the amount processed to the complex, sialic acid containing form (R_T) in terminal Golgi compartments was determined as described (Plutner et al., 1992; Tisdale et al., 1992; Davidson and Balch, 1993). The control (*A*) contained only the VSV-G expression construct.

T7 promoter (Tisdale et al., 1992). Briefly, 4 h post-infection/transfection cells were pulsed with ^{35}S-Met for 10 min. The pulse was followed by a chase for 90 min in the presence of unlabeled Met. The extent of transport of VSV-G from the ER to different Golgi compartments was quantitated by following the processing of VSV-G N-linked oligosaccharides acquired in the ER to various Golgi forms using SDS-PAGE (Tisdale et al., 1992). In HeLa cells, three distinct processed forms of VSV-G can be detected after increasing time of incubation during the chase period (Plutner et al., 1992; Tisdale et al., 1992; Davidson and Balch, 1993). One form corresponds to the endo H-sensitive form found in the ER and pre-Golgi intermediates (*S* in Fig. 7). A second form appears coincident with the transport of VSV-G to the early *cis/medial*-Golgi compartments which generates an early endo H-resistant form lacking terminal Gal and sialic acid residues (R_I in Fig. 7). Subsequently, VSV-G is processed in the terminal (*trans*) Golgi compartments to the complex forms containing Gal and terminal sialic acid (R_T in Fig. 7).

VSV-G was first cotransfected with the Sarla wild-type plasmid. As shown in Fig. 7, the extent of VSV-G detected in the *cis/medial* R_I form (17%) or the trans/TGN R_T form (60%) was identical to the control lacking the Sarla wild-type plasmid (Fig. 7 *b*). The level of expression of the Sarla wild-type and mutant proteins was generally two- to fourfold higher than the level of the endogenous Sarl pool based on Western blotting (data not shown). Thus, overexpression of wild-type Sarla neither inhibited nor stimulated transport. In contrast, overexpression of the Sarla(T39N) "GDP-bound" form strongly inhibited transport (Fig. 7 *c*). In this case, less than 20% could be detected in the R_T terminally processed form, with over 70% retained in pre-Golgi endo H-sensitive forms. A weak, but reproducible inhibition was also observed with Sarla(N134I) (Fig. 7 *d*). In a typical experiment, 32% of the VSV-G remained in the endo H–sensitive S form as compared to the control in which generally 20–22% remained in the unprocessed form after a 90-min chase. The inability of this mutant to strongly inhibit transport may reflect its instability in vivo (see Discussion).

To determine the morphological site of inhibition by Sarla(T39N) in vivo, the distribution of VSV-G was examined using indirect immunofluorescence. In this case, HeLa cells were transfected with a plasmid expressing a thermoreversible form of VSV-G (tsO45) which fails to exit the ER when cells are incubated at the restrictive temperature (39.5°C) (Lafay, 1974) (Fig. 8 *A*), but is efficiently transported to the Golgi when cells are subsequently shifted to the permissive temperature (32°C) (Fig. 8 *B, arrow*). When cells were cotransfected with tsO45 VSV-G and wild-type Sarla, transport to the Golgi was normal (Fig. 8 *B*), consistent with the inability of the wild-type protein to prevent oligosaccharide processing during transient expression. In contrast, overexpression of Sarla(T39N) by two- to fourfold potently inhibited export from the ER (Fig. 8 *C*). Quantitatively, >80–90% of the transfected cells failed to export VSV-G from the ER in the presence of Sarla(T39N). This result supports the interpretation that the role of mammalian Sarla, like that observed in yeast, is to regulate export from the ER.

Sarla Is Required for ER to Golgi Transport In Vitro

To examine the biochemical role of Sarl in transport, we utilized an assay which efficiently reconstitutes ER to Golgi and intra-Golgi transport in semi-intact cells (Beckers et al., 1987; Baker et al., 1988; Schwaninger et al., 1991; Plutner et al., 1992; Davidson and Balch, 1993). The assay was supplemented with UDP-GlcNAc to promote processing of VSV-G to the R_I endo H–resistant form (Davidson and Balch, 1993). Addition of affinity-purified antibody was

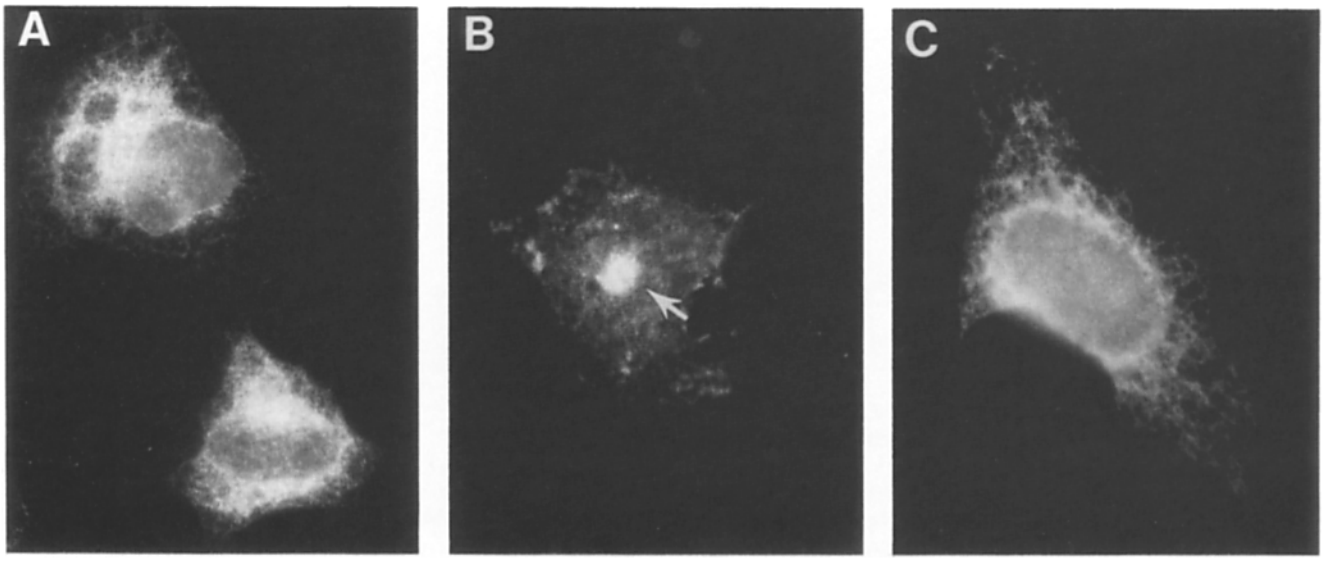

Figure 8. Transient expression of Sarla(T39N) in vivo inhibits the export of VSV-G from the ER. Vaccinia-infected HeLa cells were transfected with a plasmid expressing the temperature-sensitive (tsO45) form of VSV-G for 4 h at 39.5°C. 4 h after transfection, cells were either retained at the restrictive temperature (39.5°C) (*A*) or shifted to the permissive temperature (32°C) and incubated for 2 h in the presence of wild-type Sarla (*B*) or Sarla(T39N) (*C*) as described previously (Tisdale et al., 1992). Cells were fixed, permeabilized, and the distribution of tsO45 VSV-G determined as described previously (Plutner et al., 1992). In (*B, arrow*), VSV-G exactly overlaps with *Lens culinaris* lectin which binds principally to terminal *N*-acetylglucosamine, a marker for *cis/medial* compartments of the Golgi.

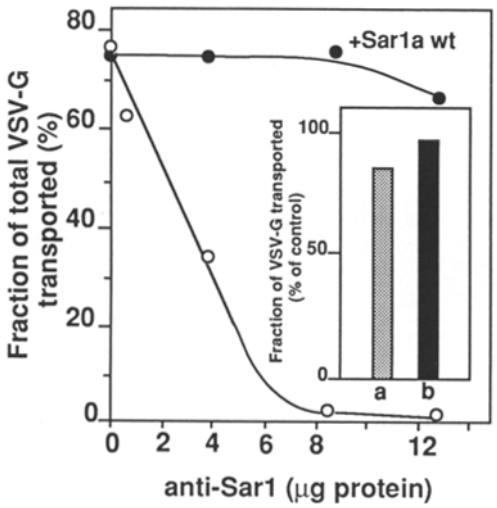

Figure 9. An antibody specific for Sar1 inhibits ER to Golgi, but not intra-Golgi transport in vitro. Semi-intact cells and cytosol were preincubated for 60 min on ice in the presence of the indicated concentration of affinity purified antibody specific for Sar1 (*open circles*). In the closed circles, Sar1a wild-type protein was added at a fivefold molar excess during preincubation on ice. Cells were subsequently transferred to 32°C, and incubated for 75 min in the presence of ATP and UDP-GlcNAc as described previously (Davidson and Balch, 1993). The amount VSV-G processed to the endo H-resistant (R_1) form was determined as described in Materials and Methods. (*Inset*) Isolated Golgi stacks were incubated in the presence of preimmune IgG (*a*) or 15 μg of Sar1 specific antibody (*b*) as described in Materials and Methods. The extent of [^{3}H]-GlcNAc incorporation is expressed as % of the control lacking additional Sar1a protein.

found to inhibit ER to Golgi transport by >90% (Fig. 8, *open circles*). Inhibition was specific since incubation of the antibody in the presence of recombinant Sar1a wild-type protein at molar excess neutralized inhibition (Fig. 9, *closed circles*). Since the antibody inhibition may result from aggregation of Sar1a in the membrane, F_{ab} fragments were prepared and also found to potently inhibit transport (data not shown). In contrast, when the effects of the antibody were tested on an assay which reconstitutes the transport of VSV-G between isolated Golgi stacks, no significant inhibition was observed (Fig. 9, *inset*). These results provide evidence that Sar1 may be a GTPase required for export of protein from the ER, but not for transport through subsequent compartments of the Golgi complex.

Given the striking effects of expression of Sar1a(T39N) on ER to Golgi transport in vivo (Figs. 7 and 8), the effect of

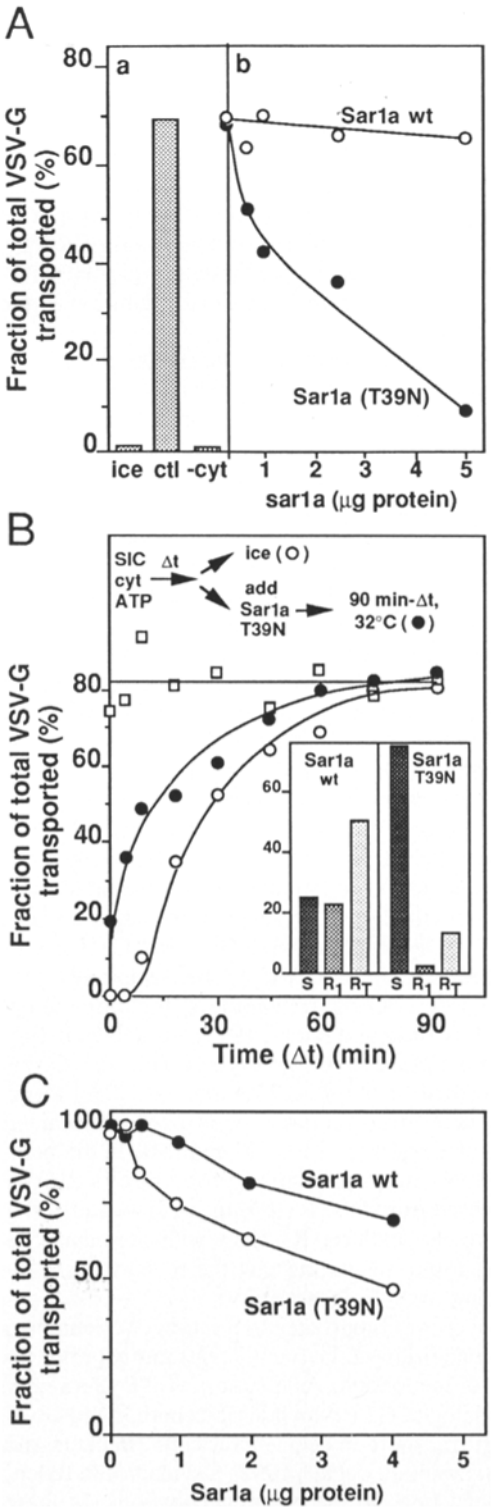

Figure 10. Sar1a T39N inhibits an early step in ER to Golgi transport in vitro. Semi-intact cells were incubated at 32°C in the presence of UDP-GlcNAc as described in Materials and Methods. (*A*) The amount of VSV-G protein processed to the endo H-resistant R_1 form in the presence (*ctl*) or absence of cytosol (*-cyt*) (*a*), or (*b*) in the presence of cytosol supplemented with increasing concentrations of the Sar1a wild-type or the Sar1a(T39N) recombinant proteins. (*B*) Semi-intact cells were incubated for increasing time (Δ*t*) at 32°C prior to transfer to ice to terminate transport (*open circles*), or supplemented with 5 μg Sar1a(T39N) for 10 min on ice prior to reincubation at 32°C for a total time of 90 min (*closed circles*). In the open squares, cells were mock treated by the addition of buffer lacking Sar1a(T39N). (*B, inset*) Semi-intact cells were incubated in the presence of either 5 μg Sar1a wild-type or Sar1a(T39N) for 75 min. The amount of VSV-G processed to either the R_1 or R_T forms in the presence of UDP-GlcNAc, UDP-Gal and CMP-SA was determined as described (Davidson and Balch, 1993). (*C*) Isolated Golgi stacks were incubated in the presence of increasing concentration of Sar1a wild-type or Sar1a(T39N) as described in Materials and Methods. The extent of [^{3}H]-GlcNAc incorporated is expressed as the % of the control level of transport lacking additional Sar1a protein.

recombinant Sarla wild-type and the T39N mutant on VSV-G transport in vitro was examined. As shown in Fig. 10 *A*, addition of increasing concentrations Sarla(T39N) inhibited transport in vitro by nearly 95%. Transport was inhibited by 50% in the presence of ~2.5 μg Sarla(T39N) with maximal inhibition above 5 μg. No inhibition was detected in the presence of an equivalent concentration of the wild-type protein (Fig. 10 *A*). The level of inhibition by the mutant was found to vary between different preparations of recombinant protein. The most active preparation inhibited transport with an IC_{50} of 0.5 μg with maximal inhibition in the presence of 1.5 μg.

Transport of VSV-G from the ER to the *cis*-Golgi compartment generally has a 15–20-min lag period during which time 40–80-nm carrier vesicles bud and target to the *cis* face of the Golgi stack (Plutner et al., 1992; Balch et al., 1994). To determine whether Sarla was required for an early step reflecting vesicle budding or a later step involved in targeting or fusion, the T39N mutant protein was added at increasing time after initiation of transport. As shown in Fig. 10 *B*, transport became rapidly (within 5–10 min) insensitive to the addition of Sarla(T39N) to the assay. For example, after only 10 min of incubation in vitro, a time-point in which Golgi processed forms of VSV-G cannot be detected, >60% of the total VSV-G transported in the control lacking Sarla(T39N) was processed to the endo H–resistant R_1 form in the presence of the mutant (Fig. 10 *B*). Therefore, Sarla is clearly recruited at a very early step in transport.

Is Sar1 required only for export of protein from the ER, or, is it required also for vesicle formation from Golgi compartments? To address this question directly semi-intact cells were incubated in vitro in the presence of the sugar nucleotide precursors UDP-GlcNAc, UDP-Gal, and CMP-SA to promote the processing of VSV-G to the terminally glycosylated (R_T) form during transport to the *trans*-Golgi compartment (Davidson and Balch, 1993). As shown in Fig. 10 *B* (inset), in the presence of wild-type Sarla, VSV-G was efficiently processed to the R_1 (22%) and R_T (50%) forms after a 90-min incubation, similar to control values obtained in the absence of exogenous Sarla. In contrast, in the presence of the T39N mutant the small proportion of VSV-G which was exported from the ER (18% of total) was quantitatively chased to the mature, R_T form with less than 5% found in the R_1 form, suggesting that the mutant was incapable of blocking intra-Golgi transport.

To pursue the above important observation, we examined the effect of the addition of the Sar1(T39N) mutant after increasing times of incubation. As a control, GTPγS[1] (a nonhydrolyzable analog of GTP which inhibits both ER to Golgi and intra-Golgi transport in semi-intact cells [Beckers and Balch, 1989; Schwaninger et al., 1992; Davidson and Balch, 1993]) was added in a parallel set of incubations. In these experiments, addition of the reagent was followed by further incubation of cells for a total time of 120 min to allow any VSV-G which had matured past a particular T39N- or GTPγS-sensitive step(s) to be processed in the subsequent Golgi compartment(s) (Fig. 11). As illustrated in Fig. 11 *A* and quantitated in Fig. 11, *B–D*, whereas GTPγS led to the accumulation of VSV-G in the R_1 form when added to early time points (see Fig. 11 *C*), addition of Sarla(T39N) consistently led to maturation of VSV-G to the R_T form with little accumulation in the R_1 form (see Fig. 11, *C* and *D*). In each

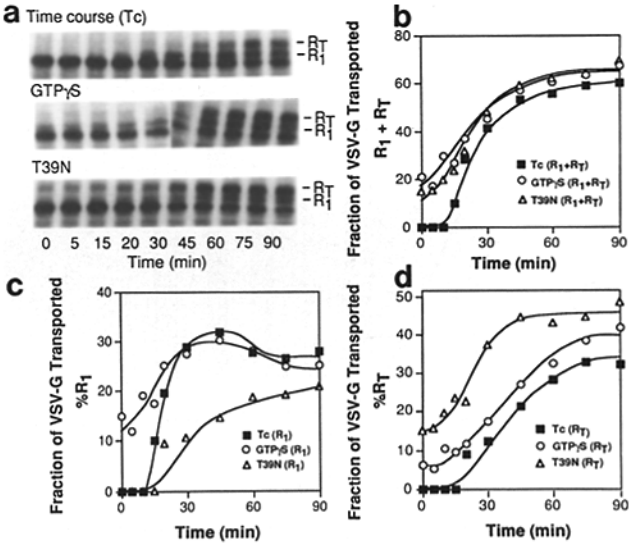

Figure 11. Sar1 is required only for export from the ER. Incubation conditions for transport of VSV-G in vitro were as described in the Materials and Methods. (*a*), (*top*) Semi-intact cells were incubated in the presence of 50 μM UDP-GlcNAc, 0.5 mM UDP-Gal, and *100* μM CMP-sialic acid for increasing time at 32°C prior to transfer to ice to terminate transport (*time course Tc*). (*Middle* and *bottom*) Cells were supplemented with 25 μM GTPγS (*middle*, GTPγS) or 1 μg Sarla(T39N) (*bottom*, T39N) at the indicated time, followed by incubation at 32°C for a total time of 120 min. (*B–D*) Quantitation of the data shown in a. (*Closed squares*) Time courses (*Tc*) for the processing of VSV-G to the R_1+R_T forms (*b*), the R_1 form (*c*) or the R_T form (*d*). (*Open circles*) The amount (% of total) of VSV-G recovered in the R_1+R_T form (*b*), the R_1 form (*c*) or the R_T form (*d*) in the presence of GTPγS added at the indicated time. (*Open triangles*) The extent of VSV-G recovered in the R_1+R_T form (*b*), the R_1 form (*c*) or the R_T form (*d*) after 120 min incubation in the presence of Sarla(T39N) (1 μg) added at the indicated time. The amount of R_1 recovered in the presence of the T39N mutant at later time points (*c*, *open triangles*) reflects the residual VSV-G which was not transported from the *cis/medial*-Golgi compartments to the *trans*-Golgi compartment in the control (*c*, *closed squares*). The amount of VSV-G processed to either the R_1 or R_T forms was determined as described in Materials and Methods.

case, the inhibitory effect of the mutant (or GTPγS) preceded processing by ~5–10 min, consistent with the notion that both reagents inhibit an early step in vesicle function. Thus, it is evident that while the general reagent GTPγS inhibits vesicle formation between multiple compartments, Sar1 is required only for export from the ER.

The effects of Sarla were also examined using an assay which reconstitutes the transport of VSV-G between isolated Golgi stacks (Balch et al., 1984). When Sarla(T39N) was added at a concentration sufficient to inhibit ER to Golgi transport by >80% (5 μg) (Fig. 10 *A*) only partial inhibition of transport was observed. In this case, inhibition was less than 25% of a control incubation containing an equivalent concentration of wild-type Sarla (Fig. 10 *C*). The latter protein also partially inhibited (by ~25%) transport. The weak inhibition of transport between isolated Golgi stacks by either the wild-type or Sarla(T39N) mutants may reflect nonspecific effects of the recombinant protein on the assay. Neither wild-type nor mutant Sarla were found to have any effect

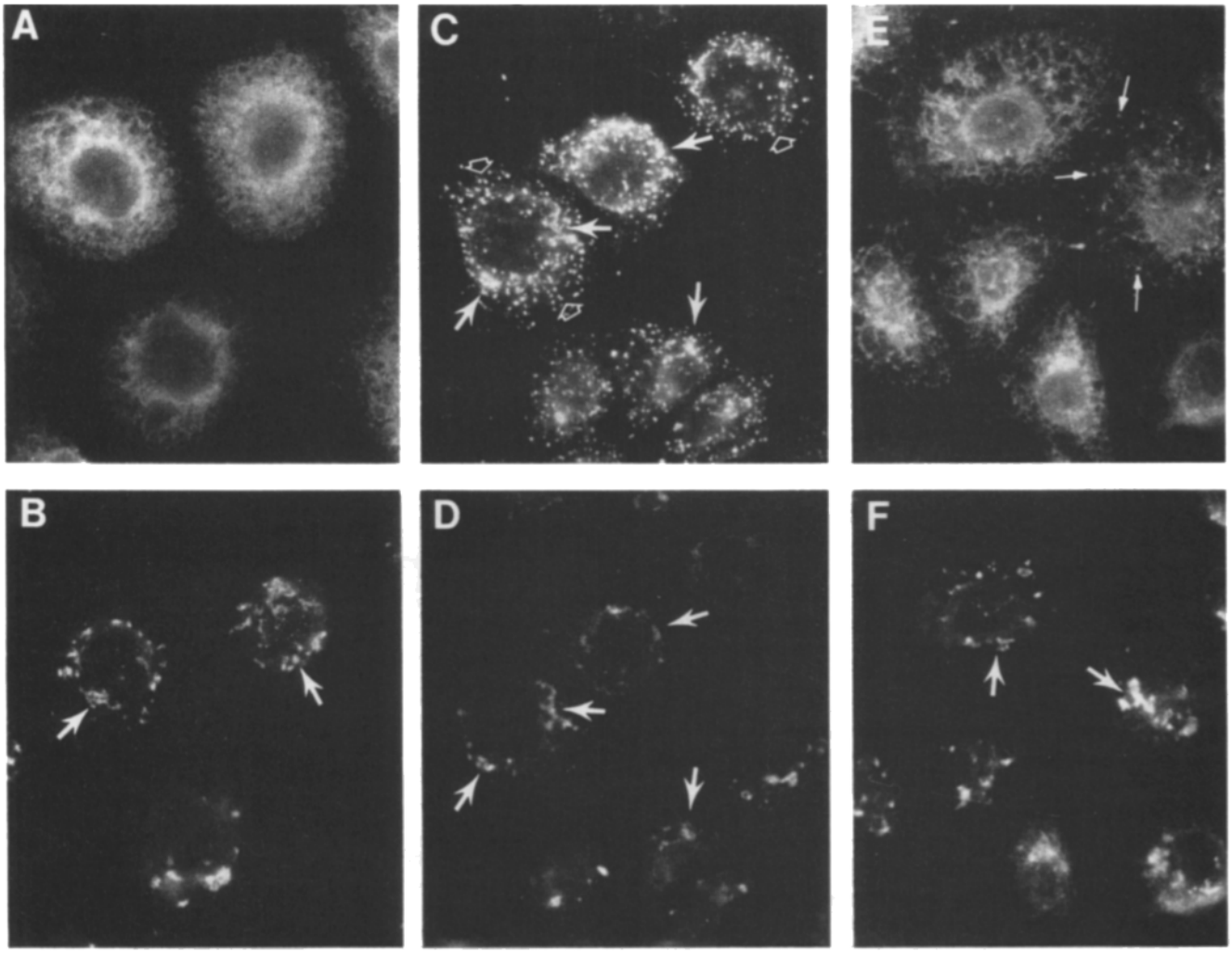

Figure 12. Sarla T39N inhibits the export of VSV-G from the ER in vitro. Cell permeabilization, incubation conditions, and morphological analysis of transport using indirect immunofluorescence were performed as described (Plutner et al., 1992). (*A* and *B*) Distribution of VSV-G (*A*) and Man II (*B, arrows*) prior to incubation in vitro. Man II is a marker protein for the *cis/medial*-Golgi compartments in NRK cells (Plutner et al., 1992; Velasco et al., 1993). (*C* and *D*) Transport of VSV-G in cells incubated for 45 min at 32°C in vitro to pre-Golgi intermediates (*C, open arrows*) and Golgi compartments (*C, VSV-G, arrows; D, ManII, arrows*) in the presence of 5 μg Sarla wild-type protein. The structures denoted by the open arrows in *C* overlap with the distribution of the pre-Golgi marker protein p58 (Saraste and Svensson, 1991) (not shown). (*E* and *F*) Distribution of VSV-G (*E*) and Golgi (*F, ManII, arrows*) in permeabilized cells incubated for 45 min at 32°C in the presence of 5 μg of Sarla(T39N). Small arrows in (*E*) denote transport of VSV-G to punctate, pre-Golgi intermediates which overlap with the distribution of p58 (not shown).

on the steady state distribution of β-COP on Golgi compartments (data not shown).

Sarla(T39N) Specifically Inhibits Budding from the ER In Vitro

To identify the step in transport between the ER and the Golgi which requires Sarl, digitonin-permeabilized cells (Plutner et al., 1992) were incubated for 45 min in the presence of the mutant protein and the distribution of tsO45 VSV-G was examined using indirect immunofluorescence. As shown in Fig. 12, prior to incubation in vitro, VSV-G is restricted to the ER (Fig. 12 *A*). As expected, incubation of cells in the presence of the Sarla wild-type protein leads to the efficient transport of VSV-G from the ER to pre-Golgi intermediates (Fig. 12 *C, open arrows*) and compartments of the Golgi stack containing the Golgi marker enzyme α-1,2 mannosidase II (compare Fig. 12, *C* and *D, arrows*). In contrast, incubation in the presence of the T39N mutant largely inhibited exit from the ER (Fig. 12 *E*). Most of the VSV-G was retained in a diffuse ER staining pattern, although some migration of VSV-G to punctate pre-Golgi could be detected (Fig. 12 *E, small arrows*), presumably reflecting the small amount of VSV-G able to escape the block at this concentration of T39N. Addition of an inhibitory concentration of the affinity-purified Sarl-specific antibody also completely prevented export of VSV-G from the ER in vitro (not shown). These data are consistent with the results observed in vivo (Fig. 7), and suggest that the Sarl GDP-bound mutant rapidly and efficiently competes with the endogenous wild-type pool for an effector molecule critical for the generation of transport vesicles from the ER.

Discussion

Sar1 Is Enriched on ER to Golgi Carrier Vesicles in Mammalian Cells

The localization of Sar1 to the transitional region of the ER provides one of several lines of evidence that it functions in ER to Golgi transport. Sar1a was enriched 20–40-fold on putative ER to Golgi carrier vesicles and nearly 10-fold in the transitional region relative to its distribution in the bulk of the ER membrane. Transitional elements are believed to be specialized sites of export of newly synthesized protein from the labyrinth of rough ER (RER), an organelle which is exceptionally abundant in insulin-secreting pancreatic cells. In contrast to Arf1 (Stearns et al., 1990) or Rab1/Ypt1p (Segev et al., 1988; Plutner et al., 1991; Pind et al., 1994), which are abundant on pre-Golgi carrier vesicles and multiple compartments of the Golgi stack, Sar1 was confined to the cisternal elements of the proximal face of the Golgi. In yeast, Sar1p has been detected using indirect immunofluorescence on perinuclear ER elements which contain Kar2p and Sec62p, and small punctate structures which are likely to be pre-Golgi intermediates, but not in Golgi compartments containing the KEX2 gene product, a *trans*-Golgi marker (Nishikawa and Nakano, 1991). Sar1p has also been detected in exaggerated ER structures which accumulate at the restrictive temperature in the presence of the temperature-sensitive sec12 and sec18 alleles, but was not detected in Golgi-like compartments which accumulate at the restrictive temperature in the presence of either the sec1 or sec7 mutant alleles (Nishikawa and Nakano, 1991). Consistent with the distribution of mammalian Sar1p, a protein which cross-reacts with an antibody specific for the yeast Sar1p-specific GAP (Sec23p) has also been localized to the transitional region of the ER in insulin-secreting cells (Orci et al., 1991*a*).

Two additional lines of evidence support a role for Sar1 in vesicle budding from the ER. First, a Sar1-specific antibody inhibited export, possibly by preventing the assembly of a Sar1-regulated coat complex. Second, the T39N mutant was a potent inhibitor of transport of VSV-G between the ER and the *cis*-Golgi compartment. While we reported inhibition of transport based on the inability of VSV-G to be processed to the endo H-resistant R_1 form, we have observed identical levels of inhibition in the processing of VSV-G to the endo D-sensitive, 5 mannose (Man_5) containing form in a CHO cell line (clone 15B) defective in modification of VSV-G NH_2-linked oligosaccharides beyond the Man_5 structure (Rowe, T. and W. E. Balch, unpublished observations). This processing intermediate immediately precedes the appearance of the R_1 form and is a hallmark for delivery of VSV-G protein to the *cis*-Golgi compartment (Beckers et al., 1987; Plutner et al., 1992; Davidson and Balch, 1993).

How does the Sar1a(T39N) mutant inhibit export? This mutation is equivalent to the S17N substitution in $p21^{H\text{-}ras}$ which disrupts the contribution of the hydroxyl group of Ser-17 to the Mg^{2+} ion involved in guanine nucleotide binding (Feig and Cooper, 1988; Farnsworth et al., 1991). This abolishes the high affinity of $p21^{H\text{-}ras}$ for GTP producing a GDP-bound form. Improper complexing of Mg^{2+} also restricts $p21^{H\text{-}ras}$ to a conformationally inactive state. The S17N mutant is believed to interfere with wild-type Ras function in vivo by serving as a competitive inhibitor for the nucleotide exchange factor (GEF), thereby preventing GDP/GTP exchange on the endogenous wild-type pool and leading to inhibition of cell growth (Farnsworth et al., 1991). The capacity of the Sar1a(T39N) mutant to efficiently inhibit export from the ER suggests that it is rapidly equilibrated with the endogenous, functional Sar1 pool. Given the altered guanine nucleotide binding properties of the Sar1a(T39N) mutant, a reasonable interpretation at this time is that the T39N mutant may compete with the wild-type protein for a Sar1a-specific GEF, interfering with the recruitment of Sar1 to the ER membrane. This interpretation is consistent with the effects of a temperature-sensitive allele of the Sar1p-specific exchange factor (Sec12p-GEF) which, in yeast leads to the proliferation of ER elements in cells incubated at the restrictive temperature (Novick et al., 1980; Nakano et al., 1988). The reduced ability of the Sar1p-GEF in the presence of the T39N mutant to support vesicle budding is consistent with the proposed role of other GEF's involved in the recruitment and activation of Arf1 (Donaldson et al., 1992*a*,*b*; Helms and Rothman, 1992; Dascher and Balch, 1994), Rab1 (Nuoffer et al., 1994), and SEC4 (Moya et al., 1993). In general, molecules promoting nucleotide exchange may play a crucial first step in the recruitment and subsequent activation of small GTP-binding proteins involved in vesicular transport between compartments of the exocytic and endocytic pathways.

The N134I substitution was considerably less potent than the T39N mutant in inhibiting ER to Golgi transport. We found this surprising given the fact that equivalent mutations in Rab1a (N124I), Rab1b(N121I), and Rab2 (N119II) are potent *trans* dominant inhibitors of ER to Golgi transport in vivo and in vitro (Tisdale et al., 1992; Pind et al., 1994). Rab1 mutants allow vesicles to bud from the ER, but prevent their fusion to the *cis*-Golgi compartment (Tisdale et al., 1992; Pind et al., 1994). In yeast, the equivalent ypt1 or sec4 mutant alleles are dominant lethal with marked secretory defects (Schmitt et al., 1986, 1988; Walworth et al., 1989). In the case of $p21^{H\text{-}ras}$, the N116I substitution destabilizes the nucleotide-binding pocket (Der et al., 1986; Feig and Cooper, 1988; John et al., 1993), resulting in a high exchange rate. This altered conformation restricts $p21^{H\text{-}ras}$ to the activated state, triggering oncogenic transformation (Walter et al., 1986). One possible explanation for the inability of Sar1a(N134I) to inhibit transport is that the folding or stability of the protein is compromised. Consistent with this interpretation, we found that expression of the Sar1a(N134I) mutant in *E. coli* leads to extensive aggregation. Unlike similar mutations in Rab1 (Nuoffer et al., 1994; Pind et al., 1994), we have been unable to purify a soluble form of the N134I mutant to test its function in vitro. Further analysis of this mutant and a mutant restricted to the GTP-form are currently under investigation.

Sar1, a GTPase Specific for Export from the ER

The striking enrichment of Sar1 on transitional carrier vesicles, and the ability of both Sar1-specific antibodies and the T39N mutant to inhibit ER to Golgi, but not intra-Golgi transport in semi-intact cells, supports the conclusion that Sar1 regulates vesicle budding at only one step of the secretory pathway-export from the ER. While the biochemical evidence for this interpretation is compelling, it is inconsis-

tent with data from a genetic analysis in yeast where SEC23 (Sar1p-GAP) function was required for at least two sequential steps: transport from the ER to the *cis*-Golgi compartment, and between early, but not late Golgi compartments (Graham and Emr, 1991). At this time there are three possible explanations for this apparent contradiction. One possibility, albeit one which we consider unlikely, is that the compartmental organization of the early secretory pathway in yeast is different from that found in mammalian cells. Alternatively, an interpretation of the genetic analysis which cannot be ruled out is that Sar1p/Sec23p are essential for delivery of factor(s) which is subsequently required for transport between early, but not late Golgi compartments (Graham and Emr, 1991). Finally, it remains to be established whether a larger Sar gene family exists, similar to that observed for Rab and which may contain many more divergent species specialized for individual stages of the exocytic (or endocytic) pathway. In this case, Sec23p may serve as a common GAP for several of these related GTPases. In any case, the presence of Sar1p on the *cis*-face of the Golgi stack is consistent with the possibility that this distribution represents its site of recycling for reuse in multiple rounds of transport.

The requirement for a unique GTPase-regulating export from the ER is intriguing given recent evidence that export of protein from the ER may involve both the sorting and concentration of cargo from resident ER proteins during vesicle budding (Mizuno and Singer, 1993; Balch et al., 1994). In contrast, transport between sequential Golgi compartments occurs without further concentration (Orci et al., 1989; Balch et al., 1994). These observations can be rationalized if the transport machinery governing export from the ER differs, at least in part, from that involved in transport between compartments of the Golgi stack. It is now recognized that coat components found on nonclathrin-coated vesicles mediating transport between Golgi compartments (Serafini et al., 1991*a*,*b*; Waters et al., 1991; Taylor et al., 1992; Orci et al., 1993; Palmer et al., 1993) as well as components involved in fusion of Golgi carrier vesicles (Wilson et al., 1989; Rothman and Orci, 1992; Söllner et al., 1993) are also required for ER to Golgi transport in mammalian cells (Beckers et al., 1989; Pepperkok et al., 1993; Peter et al., 1993; Pind et al., 1994) and in yeast (Kaiser and Schekman, 1990; Hosobuchi et al., 1992). One possible role for Sar1 may be to provide an additional level of regulatory control, perhaps related to the need to sort and concentrate cargo to ensure efficient export from the bulk of the ER (Balch et al., 1994).

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