Localization of the small GTP-binding protein rab1p to early compartments of the secretory pathway

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SUMMARY

We have studied the localization of the small GTPase rab1p in different cell types using polyclonal antibodies prepared against the rab1A isoform of the protein. Immunofluorescence microscopy of normal rat kidney (NRK) and mouse myeloma cells showed the association of the protein with the Golgi complex and peripheral sites where it colocalized with p58, a pre- and cis-Golgi marker protein. Rab1p and p58 also had similar distributions in membrane fractions derived from rat pancreas microsomes. Both were concentrated in two intermediate density subfractions between the rough endoplasmic reticulum and trans-Golgi, whereas rab6p, previously localized to middle and trans-Golgi, was enriched in the light density trans-Golgi fraction. Immunoperoxidase electron microscopy of NRK and myeloma cells revealed the association of rab1p with 1-2 cisternae, vacuolar, and tubulovesicular membranes in the cis-Golgi region. The rab1p-specific staining typically covered the entire lateral surface of the cisternae but, in weakly stained cells, local labeling between closely opposed membranes could also be seen. The rab1p-positive pre-Golgi compartment had a predominantly tubulovesicular appearance in NRK cells whereas in myeloma cells it consisted of vacuoles surrounded by rab1p-positive vesicles and tubules of heterogenous size. In both cell types the rough ER cisternae and the nuclear envelope contained negligible labeling and no continuities between these and the rab1p-positive membranes were observed. In addition, in myeloma cells the smooth ER subcompartment, containing endogenous retrovirus particles, was devoid of rab1p-labeling. These results indicate that the pre-Golgi (intermediate) compartment consists of different membrane domains and its morphology can vary considerably between different cell types. Further, they suggest that the recruitment of rab1p to membranes occurs predominantly in a post-ER location and that the protein functions in targeting/fusion events within the pre- and cis-Golgi membranes.

Key words: rab protein, ER-to-Golgi transport, pre-Golgi compartment, secretory pathway, immunoelectron microscopy

INTRODUCTION

Work carried out in the past few years has resulted in the identification of a large number of proteins as components of the molecular machinery responsible for vesicular transport in eukaryotic cells (Rothman and Orci, 1992; Pryer et al., 1992). Among these, GTPases of the Sec4/Ypt1/rab family, a branch of the p21ras superfamily, appear to have an important regulatory function (see Goud and McGaffrey, 1991; Zerial and Stenmark, 1993; Pfeffer, 1994, for reviews). In the yeast *Saccharomyces cerevisiae*, mutations in the YPT1 and SEC4 genes interrupt protein transport and membrane traffic between the endoplasmic reticulum (ER) and the Golgi complex, and the Golgi and the plasma membrane, respectively (Salminen and Novick, 1987; Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990; reviewed by Ferro-Novick and Novick, 1993). In mammalian cells, over-expression of wild-type rab-proteins or their mutated forms, displaying altered GTP/GDP-binding or GTPase activities, interferes with different steps of intracellular transport (Bucci et al., 1992; van der Sluijs et al., 1992; Tisdale et al., 1992; Nuoffer et al., 1994). In addition, the use of antibodies or antisense oligonucleotides has demonstrated the function of different rab proteins in the regulation of specific transport steps (Plutner et al., 1991; Lombardi et al., 1993; Lledo et al., 1993; Huber et al., 1993). Further, they suggest that the recruitment of rab1p to membranes occurs predominantly in a post-ER location and that the protein functions in targeting/fusion events within the pre- and cis-Golgi membranes.

Recent experiments with yeast have suggested that the proteins of the Sec4/Ypt1/rab family
may be required for the correct assembly of the v- and t-
SNARE-containing protein complexes involved in the docking
between the rough ER and the Golgi complex: rab1Ap, 
rab1Bp, and rab2p. Rab1A is the mammalian homologue of
Ypt1, as shown by its ability to functionally replace the latter
in S. cerevisiae (Haubruck et al., 1989). Mammalian cells also
contain another rab1 isoform, rab1B, which shares 92%
homology with rab1A (Vieth et al., 1990). Previous studies
using light microscopy and cell fractionation have suggested
that rab1Bp associates both with the ER and the Golgi complex
(Plutner et al., 1991). Rab2p has been localized by immuno-
electron microscopy to tubulovesicular and cisternal
membranes in the intermediate compartment/cis-Golgi region
(Chavrier et al., 1990a). Functional studies have indicated that
mutations in the GTP-binding domains of rab1 and rab2
proteins inhibit transport between the early compartments of
the secretory pathway (Tisdale et al., 1992; Nuoffer et al.,
1994).

Despite a number of studies, the organization of the boundary
between the rough ER and the Golgi complex still
remains a matter of debate (for reviews see Pelham, 1989;
Hauri and Schweizer, 1992; Saraste and Kuusmanen, 1992;
Bonatti and Torrisi, 1993; Lippincott-Schwartz, 1993;
Hendricks and Fuller, 1994). Specifically, the functions of
the pre-Golgi or intermediate compartment are not well estab-
lished and it remains unclear how many vesicular transport
steps connect the rough ER with the Golgi stacks. To study
these questions, we have examined the subcellular localization
of the rab1 protein. Results obtained using double-immunoflu-
erscence microscopy and cell fractionation demonstrated that
the protein displays a broad intracellular distribution which
resembles that of the pre- and cis-Golgi marker protein p58.
Furthermore, we have used immunoelectron microscopy to
examine for the first time in detail the localization of rab1p at
the ER-Golgi interface and within the Golgi complex. These
results are discussed in terms of the organization of the pre-
Golgi compartment and the function of rab1p in the early part
of the secretory pathway.

MATERIALS AND METHODS

Materials

Brefeldin A (BFA) was kindly provided by Dr Akira Takatsuki
(Deptartment of Agricultural Chemistry, University of Tokyo). Mouse
myeloma (RPC 5.4) cells were obtained from the American Tissue
Culture Collection (Rockville, MD). All cell culture reagents were
purchased from Gibco (Grand Island, NY), except nonessential amino
carboxylic acids that were from Flow Laboratories (Irvine, Scotland).
Nitro-
cellulose filters (0.2 μm pore size) were obtained from Schleicher &
Schuell (Dassel, Germany) and affinity-purified, 125I-labelled Protein
A from Amersham (Buckinghamshire, England). The protein assay
reagent as well as the Spurr low-viscosity embedding media for
electron microscopy were from Bio-Rad (Richmond, CA). Aprotinin
(Trasylol) was purchased from Bayer (Leverkusen, Germany) and
LC-NHS-biotin from Pierce (Rockford, IL). All other reagents were
obtained from Sigma (St Louis, MO).

Antibodies

Antibodies against rab1Ap were generated by immunizing rabbits
with purified, recombinant H-rab1Ap as described previously (Mari-
donneau-Parini et al., 1991; Baily et al., 1991). The antibodies were
affinity-purified by binding to rab1Ap-containing nitrocellulose
strips. The preparation and characterization of the rabbit antiserum
against purified rat pancreas p58 have been reported earlier (Saraste
and Svensson, 1991). The biotinylation of affinity-purified anti-p58
antibodies was carried out according to Peränen (1992). Goat anti-
rabbit IgG-TRITC conjugate was purchased from Biosoys
(Compiègne, France), HRP-coupled goat anti-rabbit F(ab)2-fragments
from Immunotech (Marseille, France), and biotinylated goat anti-
rabbit IgG as well as avidin-FITC from Zymed (San Francisco, CA).

Cell culture

Normal rat kidney (NRK) cells were grown in Dulbecco’s modified
Eagle’s medium (DME) containing 10% FCS, 2 mM L-glutamine,
penicillin (100 i.u./ml), and streptomycin (100 μg/ml). Brefeldin A
was added to the culture medium at 5 μg/ml and incubation of cells
at low temperature was carried out as described previously
(Kuismanen and Saraste, 1989). The IgG-secreting mouse myeloma
(RPC 5.4) cells were grown as a suspension culture in DME contain-
ing high glucose supplemented with 10% FCS, 1% non-essential
amino acids, 1 mM sodium pyruvate, as well as L-glutamine and the
antibiotics at the concentrations given above.

Cell fractionation

The procedures used for the isolation of rat pancreatic microsomes
and their subfractionation, by flotation in sucrose gradients, have been
described previously (Saraste et al., 1986). After centrifugation the
four visible bands (B1-B4; see Lahtinen et al., 1992) were collected,
their sucrose concentrations were adjusted to 0.3 M, and the
membranes were concentrated by centrifugation for 90 minutes at
100,000 gav. The pelleted membranes were resuspended in 0.25 M
sucrose containing 100 i.u./ml aprotinin, 1 mM PMSF, and 10 μg/ml
each of soybean trypsin inhibitor, chymostatin, leupeptin, antipain,
and pepstatin. The membranes were divided into aliquots, frozen in
liquid nitrogen, and stored at –80°C.

Quantitation of rab proteins in cell fractions

Samples of the different fractions (corresponding to 30 μg of protein)
were solubilized in SDS-sample buffer. The proteins were separated
by SDS-PAGE using 5-15% gradient gels and thereafter transferred
to nitrocellulose. Immunostaining with anti-rab antibodies followed by
125I-labelled Protein A was carried out as described previously
(Lahtinen et al., 1992). The primary antibodies were used at dilutions
that gave linear detection of the respective antigens (rab1p and rab6p).
Finally, the nitrocellulose filters were dried and used to expose Kodak
X-Omat film. The immunoreactive protein bands were located with
the help of the developed film, whereafter the corresponding nitro-
cellulose pieces were excised and counted in a gamma-counter.

Immunofluorescence microscopy

The procedures used for fixation, permeabilization, and antibody
incubation of cultured cells have been described previously (Goud et al.,
1990; Saraste and Svensson, 1991). For double-staining NRK cells
were first incubated with affinity-purified anti-rab1Ap IgG followed
by TRITC-coupled goat anti-rabbit IgG. After extensive washing the
unreacted anti-rabbit antibodies were blocked by incubating the cells
with 1% normal rabbit serum in PBS. In the second step the cells were
stained with affinity-purified, biotinylated anti-p58 antibodies
followed by FITC-avidin. No cross-reactivity was observed between
the two detection systems, which also gave similar staining intensi-
Oties. To quantify the overlap in the peripheral, spotty staining obtained
with the two antibodies, photographs were taken from two experi-
ments (including control, low temperature- and BFA-treated cells)
using identical exposure times for the two fluorochromes. For each
experiment, the easily discernible immunoreactive spots outside the
Golgi region were counted from 15-20 cells and the staining overlap
was determined using a transparent sheet and a marker pen of different
colour for each fluorochrome. In the case of mouse myeloma cells, the cells grown in suspension were first washed once with PBS at 37°C and then attached to microscope slides by cytocentrifugation for 3 minutes at 1,200 rpm in Labofuge A (Heraeus). The cells were fixed immediately after centrifugation, permeabilized, and incubated with 2% goat serum in PBS to inhibit nonspecific binding of antibodies. The cells were then incubated with affinity-purified anti-rab1Ap antibodies, followed by biotin-coupled goat anti-rabbit IgG and avidin-FITC. After mounting the cells were examined in a Leitz Diaplan microscope and photographed on Kodak Tri-X Pan film (400 ASA).

Immunoelectron microscopy
Before fixation the myeloma cells were washed with PBS and attached to plastic culture dishes coated with poly-L-lysine. The pre-embedding immunoperoxidase procedure used for staining of cells with affinity-purified anti-rab1Ap and goat anti-rabbit F(ab)2-fragments coupled HRP has been described in detail elsewhere (Saraste et al., 1987; Saraste and Svensson, 1991; Goud et al., 1990). Thin sections of the immunolabelled cells, embedded in low-viscosity Spurr resin were stained with lead citrate and examined in JEOL 100 CX or 100 CX II electron microscopes operated at 60 kV.

RESULTS

Overlapping distributions of rab1p and p58
Affinity-purified anti-rab1p antibodies were isolated from the serum of a rabbit immunized with purified, bacterially expressed H-rab1A protein (Maridonneau-Parini et al., 1991; Bailly et al., 1991). In immuno-overlays these antibodies cross-reacted with bacterially expressed rab1Bp (data not shown) and have also been shown to detect both rab1 isoforms in mammalian cells (Huber et al., 1994).

In immunofluorescence microscopy the anti-rab1Ap antibodies gave a strong, juxta-nuclear, Golgi-like staining both in NRK and mouse myeloma cells. In both cell types the protein was also detected in a number of punctate structures, present in the Golgi region as well as more peripheral parts of the cytoplasm (Fig. 1A,B; Fig. 2). In NRK cells some weak, diffuse staining was seen, which is most likely due to the cytoplasmic pool of the protein (Bailly et al., 1991). Double-immunofluorescence staining of NRK cells showed a consid-

Fig. 1. Colocalization of rab1p and p58 in the central Golgi region and peripheral pre-Golgi sites in NRK cells. The cells were grown and fixed at 37°C, permeabilized, and stained with affinity-purified antibodies against rab1Ap, followed by anti-rabbit IgG-TRITC conjugate (A and B). Subsequently, the cells were double-stained with affinity-purified, biotinylated anti-p58 antibodies followed by avidin-FITC (C and D). The arrows and the boxed areas indicate colocalization of the two proteins in the peripheral pre-Golgi elements. The arrowheads in B and D indicate one such site which is positive for p58 but negative for rab1p. Bars, 10 μm.
erable overlap between the observed rab1p localization (Fig. 1A,B) and the staining obtained with antibodies against p58 (Fig. 1C,D), a pre-Golgi compartment and cis-Golgi marker protein (Saraste et al., 1987; Saraste and Svensson, 1991). These results clearly indicated that the two proteins are located in the same or closely associated membranous structures in the Golgi region (compare Fig. 1B,D). In addition, determination of the number and overlap of the peripheral, punctate structures stained by the two antibodies, indicated that rab1p was present in about 70% of the p58-positive pre-Golgi structures (Fig. 1, see also Materials and Methods).

To further compare the localizations of rab1p and p58 in the early part of the secretory pathway, we used double-immunofluorescence microscopy to examine the effects of low temperature (16°C) and brefeldin A (BFA) on the distribution of these two proteins in NRK cells. Both these treatments are known to inhibit ER-to-Golgi transport (Saraste and Kuismanen, 1984; Takatsuki and Tamura, 1985). At 16°C the transport inhibition correlates with the accumulation of the p58-positive, vacuolar pre-Golgi intermediates in the periphery of the cells. In BFA-treated cells p58 is redistributed to rough ER-associated, tubulovesicular membranes, scattered throughout the cytoplasm (Saraste and Svensson, 1991).

The colocalization of rab1p and p58 in the Golgi region and peripheral membranes was maintained in cells incubated for 120 minutes at 16°C (Fig. 3A,B). In the low temperature-treated cells about 95% of the p58-positive, peripheral spots also contained rab1Ap. This may indicate that low temperature incubation increases the membrane-bound pool of this protein and thereby improves its detectability in the peripheral membranes. In cells treated for 60 minutes with BFA (Fig. 3C,D) about 80% of the p58-positive, punctate structures were also reactive with anti-rab1Ap antibodies. In contrast to both steady-state and 16°C-treated cells, a considerable number, about 25%, of the rab1p-positive sites were negative for p58, which may suggest that partial segregation of the p58- and rab1p-containing membranes takes place in the drug-treated cells. However, since the polyclonal antibody is likely to detect
both rab1Ap and rab1Bp, the possibility exists that this observed non-overlap is due to differential effect of BFA on the distribution of the two isoforms. In this context, we have also noted that p58 and rab1p have partially dissimilar localizations in myeloma cells undergoing mitosis (J.S., unpublished results).

We also examined the intracellular distribution of rab1p by cell fractionation using well characterized rat pancreas subfractions obtained by equilibrium centrifugation of total microsomes in sucrose density gradients. Previous marker analysis has shown that these fractions are enriched in the successive membranes of the early part of the secretory pathway, with the B1 and B4 fractions representing the rough ER and middle/trans-Golgi, respectively (Saraste et al., 1986; Lahtinen et al., 1992). Immunoblotting with $^{125}$I-labelled Protein A demonstrated the enrichment of rab1p in the two intermediate density subfractions B2 and B3 (Fig. 4). The overall distribution of the protein was similar to that observed for p58 (Fig. 4; Lahtinen et al., 1992). However, in contrast to p58, a considerable amount of rab1Ap was detected in the light density, middle/trans-Golgi fraction (B4). This result may be partly due to the overlap between the different fractions but can also reflect differences in the distributions of the two proteins within the Golgi complex of the pancreatic cells. Namely, we have observed that in NRK cells p58 is predominantly found in large vacuolar elements in the cis-Golgi region and is only occasionally detected in one cis-Golgi cisterna (Saraste and Svensson, 1991). In contrast, in the same cells rab1p is invariably localized to 1-2 cisternae on the cis-side of the Golgi stacks (see below).

Analysis of the pancreas subfractions also provided the possibility to compare within the Golgi complex the distribution of rab1p with that of rab6p, another small GTPase which has previously been localized to the middle and trans-Golgi/TGN and post-Golgi membranes in other cell types (Goud et al., 1990; Antony et al., 1992; Deretic and Paparnaster, 1993; Tixier-Vidal et al., 1993). As shown in Fig. 4, rab1p and rab6p were well separated in these gradients, with the latter being enriched in the light density trans-Golgi fraction (B4). This result is in accordance with the suggestion that rab1p and rab6p function in successive transport steps within the Golgi complex (Goud et al., 1990; Martinez et al., 1994).

**Immunoelectron microscopic localization of rab1p**

**Central Golgi complex**

To study the distribution of rab1p at the ultrastructural level we applied the pre-embedding immunoperoxidase method that was previously used to localize rab6p to the cytoplasmic surface of middle- and trans-Golgi cisternae in the NRK cells (Goud et al., 1990). Intensive and specific reaction could be seen in the Golgi complex of NRK (Figs 5-7, 8A) and myeloma cells (Fig. 9A) after staining with anti-rab1Ap antibodies and goat anti-rabbit F(ab)$_2$-peroxidase. In all the cells examined the polarized staining was confined to 1-2 cisternae at one side of the Golgi stacks (Figs 5-8) and remained polarized over several serial sections studied (Figs 5-7). The staining was distributed over the whole lateral surface of the positive cisterna whereas the often dilated ends of the cisternae were negative (Figs 7, 8). In addition, in cells displaying weaker staining of the Golgi complex, more localized deposits of peroxidase reaction product could be seen between membranes (Fig. 7).

The above immunofluorescence and cell fractionation results showed that the overall intracellular distribution of rab1p is similar to that of p58, which has previously been localized to the cis-aspect of the Golgi in several cell types (Saraste et al., 1987; Saraste and Svensson, 1991; Lahtinen et al., 1992). The most convincing demonstration of the association of rab1p with the cis-side of the Golgi stacks was obtained in the case of mouse myeloma cells, in which the well organized, often circular Golgi complex displays a clear cis-trans polarity (Fig. 9A). In addition, in both NRK and myeloma cells the labeled cisternae had a fenestrated morphology (Figs 6-8), which is a characteristic of the cis-Golgi cisternae.

In thin sections the morphologically less organized, reticular Golgi complex of NRK cells often appears as multiple cisternal stacks with adjacent stacks frequently showing opposite polarity (Figs 5,6). Interestingly, serial section analysis indicated that the different stacks can share the same rab1p-positive cis-Golgi elements (Fig. 6). This indicates that in addition to tubular trans-Golgi membranes (Roth et al., 1985; Cooper et al., 1990), also cis-Golgi elements can provide interconnections between the different stacks of the Golgi complex.

In addition to Golgi cisternae, rab1p was present in
tubulovesicular membranes at the cis-side of the Golgi complex (Fig. 5, insets; Figs 8,9A). The morphology of these Golgi stack-associated membranes shared similarities to the rab1p-positive membranes seen at the periphery of the cells (Figs 8,9; see below). In both cell types studied they were seen as extensive, often discontinuous membrane clusters at the cis-side of the Golgi. In addition to vesicular and tubular membranes, they frequently contained also pleiomorphic or vacuolar elements (Fig. 9A,B).
Localization of rab1p to pre- and cis-Golgi membranes

Pre-Golgi membranes

Immunoelectron microscopy of NRK cells showed that the peripheral, rab1p-positive sites, seen by immunofluorescence microscopy (Fig. 1A,B), represent clusters of tubular and vesicular membranes (Fig. 8). These structures were widely distributed in the cytoplasm and were encountered even close to the plasma membrane (Fig. 8A,B). Frequently, the rab1p-containing membranes were seen in close to the rough ER cisternae or the nuclear envelope (Fig. 8A,C), but no direct continuities between these membranes could be observed. Although predominantly tubulovesicular in overall morphology, many of the clusters also contained larger, pleiomorphic or vacuolar elements (Fig. 8). Based on the colocalization of rab1p and p58 at these peripheral sites (Fig. 1), it can be assumed that these correspond to the pleiomorphic pre-Golgi intermediates to which p58 has been previously localized in NRK cells (Saraste and Svensson, 1991).

The distribution of the rab1p-positive, peripheral membranes in mouse myeloma cells was similar to that seen in NRK cells (Figs 2,9). However, morphologically these myeloma cell membranes differed considerably from those seen in NRK cells, consisting of large vacuolar elements (diameter 200-500 nm), surrounded by clusters of tubulovesicular membranes (Fig. 9C-H). Some of the vacuoles contained membranous material in their lumen (Fig. 9B,C), as observed previously in the case of pre-Golgi vacuoles in BHK21 cells (Saraste and Kuismanen, 1984). The amount of the vacuole-associated membranes varied from a few vesicles or tubules...
(Fig. 9C,E,F) to often extensive membrane clusters (Fig. 9G,H). These tubulovesicular elements were heterogeneous in size, varying usually between 60 and 100 nm in diameter, but also included a population of unexpectedly small, about 30 nm vesicles (Fig. 9B,C; small arrows). It was apparent from many of the images (see e.g. Fig. 9H) that the bulk of rab1p within the pre-Golgi membranes is associated with the cytoplasmic surface of these vacuole-associated tubulovesicular membranes.

Endoplasmic reticulum

In previous light microscopic studies rab1Bp was localized throughout the entire reticular ER network in NRK cells (Plutner et al., 1991). In contrast, in our immuno-electron microscopic studies of NRK and myeloma cells, stained with the anti-rab1Ap antibodies (which detect both isoforms of the protein), we observed negligible labeling of the RER cisternae and the nuclear envelope (Figs 5-9). Occasional, apparent labeling of these membranes was restricted to sites adjacent to the rab1p-positive, tubulovesicular membrane clusters and is likely to be due to the diffusion of the peroxidase reaction product from the strongly reactive smooth membranes (see e.g. Fig. 9H). Also, at these locations the RER cisternae and the nuclear envelope were not observed to contain budding profiles.

The myeloma cells also contain a smooth ER subcompartment, which is continuous with the rough ER cisternae and represents the intracellular site of accumulation of endogenous retrovirus particles (Ottosen et al., 1980). Like rough ER, these virus-containing membranes have a widespread distribution in these cells and, in addition to the cis-Golgi region (Fig. 9A, arrowheads), were also seen in the vicinity of the peripheral, rab1p-positive pre-Golgi membranes (Fig. 9E-G, arrowheads). Irrespective of their intracellular location, negligible labeling of these membranes was observed.

DISCUSSION

We describe here the detailed localization of rab1Ap, a small GTP-binding protein which, based on genetic and in vitro studies, has been suggested to be one of the key components of the transport machinery that operates in the early part of the secretory pathway (Plutner et al., 1991; Tisdale et al., 1992; Davidson and Balch, 1993). Mammalian cells also contain another isoform, rab1Bp, which shares 92% sequence homology with the rab1A protein (Touchot et al., 1987; Zahraoui et al., 1989; Vielh et al., 1989; Chavrier et al., 1990b). The present antibodies, prepared against bacterially expressed human rab1Ap, were observed to react with both isoforms of
Localization of rab1p to pre- and cis-Golgi membranes

Fig. 9. Localization of rab1p in mouse myeloma cells. (A and B) Localization of the protein in vacuolar, tubulovesicular, and cisternal elements at the *cis* face of the Golgi complex (GC). The arrows in A indicate distinct, large membrane clusters consisting of vacuolar membranes surrounded by extensive arrays of tubules and vesicles. Two such vacuoles are shown in B in higher magnification. (C-H) Distribution and morphology of rab1p-positive, peripheral membranes in myeloma cells. D is a low magnification field showing two peripheral membrane clusters, one close to the nuclear membrane and another in the vicinity of the plasma membrane (PM). At higher resolution (C,E-H) these membranes can be seen to consist of vacuolar domains (v) surrounded by clusters of tubules and vesicles. They were frequently found close to rough ER cisternae (ER; E,F,H) and/or the nuclear membrane (G,H) and were variable in size: small (E,F), extensive (D,H). The arrowheads in (A,D-F) indicate smooth membranes, containing endogenous retrovirus particles, that are not labeled with anti-rab1p antibodies. N, nucleus; M, mitochondrion. Bars: 1 µm (A); 0.5 µm (B-H).
the protein. Studies on the expression of mutated forms of rab1Ap and rab1Bp have demonstrated that the two proteins are functionally interchangeable in ER-to-Golgi transport (Tisdale et al., 1992; Nuoffer et al., 1994). Therefore, their intracellular distributions are expected to be identical in interphase cells. However, since only rab1Ap contains the consensus sequence required for phosphorylation by the mitotic p34cdc2 kinase (Bailly et al., 1991), it is possible that the two proteins may have divergent functions during cell division.

**Subcellular localization of rab1p**

In previous immunofluorescence experiments certain monoclonal antibodies against rab1Bp were shown to give rise to a reticular, ER-like staining pattern (Plutner et al., 1991). Based on this localization data and the inhibitory effects of antibodies on VSV G-protein transport, observed using in vitro assays, it has been suggested that rab1p functions in the early budding event during the exit of transported proteins from the rough ER (Plutner et al., 1991; Schwaninger et al., 1992). In the present study, however, using a sensitive immunoperoxidase method in electron microscopy, we did not observe such a localization of the protein in the rough ER cisternae or the nuclear envelope. Therefore, the present results raise the possibility that rab1p does not function in the initial ER exit step but its membrane association occurs later along the pathway, predominantly at the level of post-ER vesicles/tubules and elements of the pre-Golgi compartment. That the recruitment and function of rab1p map in a post-ER location is also suggested by results showing that, in the presence of different mutated forms of the protein, the initial ER exit still takes place and the arrest in VSV G-protein transport occurs in punctate pre-Golgi structures (Tisdale et al., 1992; Nuoffer et al., 1994; Pind et al., 1994).

In vitro studies have further suggested that in mammalian cells vesicle budding from the ER, i.e. the appearance of G-protein in punctate pre-Golgi intermediates, involves β-COP containing coatamer complexes and that rab1Bp associates with these coat structures (Peter et al., 1993). In contrast, studies of coatamer function using microinjection (Pepperkok et al., 1993) and immunolocalization with anti-β-COP antibodies (Duden et al., 1991; Oprins et al., 1993; Hendriks et al., 1993; Krijnse-Locker et al., 1994; see also Saraste and Kuismanen, 1992, for a review) have suggested that they mainly associate with the intermediate compartment and cis-Golgi membranes. Therefore, similarly as reported in yeast cells (Salama et al., 1993; Barlowe et al., 1994), the β-COP coats are likely to operate in a later stage of transport in mammalian cells. A recent study suggested that they may be involved in protein recycling at the ER-Golgi interface (Cosson and Letourneur, 1994).

BFA is known to cause rapid and complete dissociation of the β-COP coats from membranes (Donaldson et al., 1990). The present immunofluorescence results, however, showed that in the drug-treated cells a considerable part of rab1p remained membrane-bound and redistributed together with the pre-Golgi compartment/cis-Golgi marker p58. This finding does not rule out the participation of rab1p in the assembly and/or function of the β-COP coats (Peter et al., 1993) but, together with the electron microscopic localization data, suggests that at least part of rab1p is associated with non-coated membrane surfaces.

**Organization of the pre-Golgi membranes**

Morphological and biochemical studies have demonstrated the complexity of the membranes at the ER-Golgi interface and resulted in the concept of a pre-Golgi or intermediate compartment, located between these organelles. Different views on the structural organization of this compartment and its function in ER-Golgi communication have been presented (Warren, 1987; Pelham, 1989; Hauri and Schweitzer, 1992; Bonatti and Torrisi, 1993; Lippincott-Schwartz, 1993; Hendriks and Fuller, 1994; Krijnse-Locker et al., 1994). These include the proposal that the pre-Golgi intermediates are structurally and functionally analogous to endosomes, consist of distinct vacuolar and tubulovesicular domains, and are capable of protein sorting to the forward and recycling transport pathways (Saraste and Kuisman, 1984, 1992). In contrast, a recent study on the localization of VSV G-protein in digitonin-permeabilized NRK cells concluded that these membranes simply represent a collection of ER-derived vesicles and tubules on their way to the cis-Golgi (Balch et al., 1994; Pind et al., 1994). Based on the present results, this discrepancy can be explained by the heterogeneity and morphological variability of the pre-Golgi compartment, based on the existence of subdomains, as well as by the use of a marker which can be expected to be enriched in a certain part of this compartment. Also, it should be noted that the in vitro conditions result in extensive depolymerization of the cytoskeleton, including microtubules (Plutner et al., 1992), which are known to have an important function in maintaining the structural and spatial arrangement of the early compartments of the secretory pathway (see Saraste and Thyberg, 1995, for a review).

The present results on the localization of rab1p in NRK and myeloma cells are in agreement with previous studies suggesting that the pre-Golgi compartment is composed of distinct vacuolar and tubulovesicular domains (Saraste and Kuisman, 1984; Saraste and Svensson, 1991; Lahtinen et al., 1992). The vacuolar subcompartment was particularly pronounced in myeloma cells which may correlate with their high activity in immunoglobulin synthesis and secretion. Immunoelectron microscopy indicated that rab1p can be present on the cytoplasmic surface of these pre-Golgi vacuoles. In both cell types studied, however, the protein appeared to be predominantly associated with the tubulovesicular part of the pre-Golgi membranes. Immunoelectron microscopic double-localization of rab1p with other markers is required to define the precise transport step(s) in which these rab1p-positive vesicles and tubules operate. However, together with previous results (Pind et al., 1994), the present localization data supports the idea that Rab1p, like its yeast counterpart Ypt1p (Rexach and Schekman, 1991; Segov, 1991), is involved in targeting (docking) or fusion events in the pre- and cis-Golgi membranes. Furthermore, the dual localization of rab1p to both peripheral pre-Golgi elements and central cis-Golgi cisternae emphasizes the functional relatedness of these spatially and morphologically different membranes.

The myeloma cells, like many other cultured lymphoid cell lines, contain a smooth ER compartment which represents the site of accumulation of endogenous retrovirus particles. The lack of rab1p association with this compartment indicates that
it lies proximal to the pre-Golgi compartment along the ER-Golgi pathway. In this respect it could be analogous to the smooth-ER compartment that has been described in transfected CHO cells expressing the rubella-virus E1 protein (Hobman et al., 1992).

**Role of rab GTPases in intra-Golgi transport**

In the Golgi complex rab1p displayed a clearly polarized distribution. In addition to tubulo-vesicular and vacuolar membranes, the protein was detected on the cytoplasmic surface of 1-2 cisternae at the cis-face of the Golgi stacks. The fact that the inner surface of the second, non-fenestrated cis-cisterna was also frequently stained rules out the possibility that the labeling of the Golgi cisternae was due to diffusion of the peroxidase reaction product from the nearby vacuolar and tubulovesicular membranes. In general, the staining covered the whole lateral surface of the positive cisternae, a pattern similar to that observed for rab6p, previously shown to be associated with middle and trans-Golgi cisternae in NRK cells (Goud et al., 1990). Functional studies have suggested that rab1p is required for transport between cis- and middle-Golgi membranes (Plutner et al., 1991; Nuoffer et al., 1994), whereas rab6p is involved in transport between cis/middle and trans/TGN Golgi compartments (Martinez et al., 1994). The data on the localization of rab1p and rab6p in the Golgi complex indicates that the two proteins regulate successive transport steps in the early part of the secretory pathway. The determination of their precise functions is expected to considerably advance our knowledge of the mechanisms and pathways of membrane trafficking to and within this organelle.

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