Identification of rabaptin-5, rabex-5, and GM130 as putative effectors of rab33b, a regulator of retrograde traffic between the Golgi apparatus and ER

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Abstract The role of rab33b, a Golgi-specific rab protein, was investigated. Microinjection of rab33b mutants stabilised in the GTP-specific state resulted in a marked inhibition of anterograde transport within the Golgi and in the recycling of glycosyltransferases from the Golgi to the ER, respectively. A GST-rab33b fusion protein stabilised in its GTP form was found to interact by Western blotting or mass spectroscopy with Golgi protein GM130 and rabaptin-5 and rabex-5, two rab effector molecules thought to function exclusively in the endocytic pathway. A similar binding was seen to rab1 but not to rab6, both Golgi rabs. In contrast, rab5 was as expected, shown to bind rabaptin-5 and rabex-5 as well as the endosomal effector protein EEA1 but not GM130. No binding of EEA1 was seen to any of the Golgi rabs. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Golgi; Recycling; Mass spectroscopy; Rab1; Rab5; Rab6; Rab33b; Rabaptin-5; Rabex-5

1. Introduction

The role of small GTPases belonging to the rab family in membrane trafficking has been investigated extensively. Of the 40 or so rab proteins identified biochemically to date, some have been shown to actively regulate protein transport, both in the exocytic and the endocytic pathway (for review, see [1,2]). Several rab proteins have been mapped to the secretory pathway and shown to promote various aspects of transport and membrane biogenesis. Rab1, for example, was identified first in yeast as ypt1 [3,4]. It is involved in endoplasmic reticulum (ER) to Golgi transport [5–7]. Rab6 localises to the *medial* to *trans* Golgi and promotes recycling from the Golgi to the ER [8–10]. The recently described rab33b is a novel rab protein that localises to the *medial* Golgi [11] and is unknown in its function and protein interactions.

Rabs serve as regulatory factors in the recruitment and assembly of effector molecules on membranes. Rab1 interacts with p115 and GM130 [12–14]. Within the Golgi apparatus, p115 and GM130 as well as giantin are examples of stacking and tethering proteins that are thought to keep Golgi cisternae together or tether vesicles to acceptor Golgi membranes. GM130 is found preferentially in the *cis* Golgi. It is bound to Golgi membranes via GRASP65, a stacking protein, and both GM130 and giantin have binding sites for p115 [15–21]. Rab6 interacts with rabkinesin-6 implicating it in the regulation of motility and/or membrane extensions [10,22]. Rab5 has been shown to interact with EEA1, rabaptin-5, rabaptin-5β and rabex-5. These proteins play crucial roles in docking and fusion of endosomal membranes [23–29]. Rab5 also regulates the motility of endosomes through unknown protein interactions [30]. The available data suggest that rab proteins associated with the Golgi apparatus have relatively specific sets of effector proteins.

In the present work, we tested the in vivo importance of rab33b in the organisation of the Golgi apparatus and then asked in vitro if rab33b displayed a unique or overlapping set of interacting proteins when compared with rab1, rab6 and rab5. We show by overexpression that rab33b and its GTP-bound mutant play an important role in Golgi organisation and retrograde transport. In a screen for interacting components using mass spectroscopy and Western blotting, we found that rab33b interacted in a GTP-specific manner with the Golgi protein GM130, and also with rabaptin-5 and rabex-5, two proteins that until now have been thought to function exclusively in the endosomal pathway. We also found that rab1 but not rab6 interacted with rabaptin-5 and rabex-5. We suggest that overlapping combinatorial effector sets are assembled by Golgi-associated rabs.

2. Materials and methods

2.1. Antibodies

Affinity-purified monoclonal antibodies against rab33b have been described previously [11]. Monoclonal antibodies against rab5, EEA1 and rabaptin-5 were from Transductions Laboratories (Lexington, KY, USA). Polyclonal rabbit antibodies to GM130 were a gift from Hiro Nakamura (Kanazava University, Kanazava, Japan). Polyclonal rabbit antibodies to gp27 and the cytoplasmic domain of VSV-G protein have been described previously [31,32]. Monoclonal antibodies to the lumenal domain of VSV-G protein were a kind gift from Rainer Pepperkok (EMBL, Germany). Polyclonal sheep anti-TGN46 antibodies were a gift from Vas Ponnambalam (University of Dundee, Dundee, UK). Monoclonal mouse anti-myc antibodies were a kind gift from Eric Karsenti (EMBL, Germany). Donkey anti-sheep HRP,

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goat anti-rabbit HRP, goat anti-mouse HRP, Cy3-conjugated donkey anti-mouse and anti-rabbit IgG antibodies were from Dianova (Hamburg, Germany). Donkey anti-sheep IgG (Alexa 488), goat anti-mouse IgG (Alexa 488) and goat anti-rabbit IgG (Alexa 488) antibodies were from Molecular Probes (Leiden, The Netherlands).

2.2. cDNAs

Full-length cDNA encoding rab33bwt in pBluescript KS+ has been described previously [11]. Constructs of rab33b$_{Q92L}$ and rab33b$_{T47N}$ were generated by PCR-based mutagenesis [33]. The sequences of amplified cDNA fragments were verified and cloned into pBluescript KS+. Inserts were then ligated into pCMUIV [34] and pEYFP-C1 (Clontech, Heidelberg, Germany). Rab6wt in pCMUIV has been described [8]. Rab5$_{Q79L}$ in pGEM-1 and rab1wt in pET-11d were kindly provided by Marino Zerial (Max-Plank-Institut, Dresden, Germany). PCR was performed to introduce flanking *Bam*HI sites and an upstream Kozac consensus sequence, using rab5$_{Q79L}$ or rab1wt complementary sequences as templates. The PCR product of rab5$_{Q79L}$ was cloned into pCMUIV for mammalian expression and the ORFs of rab1wt, rab6wt and rab33bwt were cloned into pGEX-4T-3 for prokaryote expression of GST-rab fusion proteins. GST-rab5wt in pGEX-5X-3 was kindly provided by Marino Zerial (Max-Plank-Institut).

2.3. Cell culture

HeLa cells were cultured on 10 mm glass coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (PAA laboratories, Linz, Austria), 10 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Eggenstein, Germany). The stable HeLa cell lines expressing GalNAc-T2-GFP or murine mannosidase II-VSV (Mann II) have been described [35,36]. For rab33bwt, rab33b$_{Q92L}$ and rab33b$_{T47N}$ in pEYFP-C1, 20 μg purified plasmid were transfected into 10 cm Petri dishes and selected for stable expression as described [37] with the modification that 3 weeks post-transfection, cells were sorted using a fluorescent-activated cell sorter (FACS) to select for YFP expression in the medium range. Stable cell lines were grown as described above but with the addition of 300 μg/ml G418 (Gibco).

2.4. Microinjections and Cy3 Shiga-like toxin-B-fragment uptake

Purified plasmids were microinjected into cell nuclei with minor modifications of previous procedures [36]. DNA concentrations ranged from 200 to 1000 ng/μl for rab33b plasmids. pCMUIV plasmids encoding pSARA (Sar1p^{dn} dominant negative mutant which blocks ER exit [36]) or VSV-G$_{tso45}$-GFP were microinjected at a DNA concentration of 200 ng/μl. A coinjection marker was used, 70 kDa fixable Cascade blue dextran (Molecular Probes, Eugene, OR, USA), at a concentration of 3.33 mg/ml. Cells were microinjected at room temperature with plasmid encoding VSV-G$_{tso45}$-GFP protein in the absence or presence of plasmid encoding rab33b, incubated at 39.5°C for 6 h, and then shifted to the permissive temperature, 31.5°C. In experiments assessing the effect of rab33b mutants on retrograde trafficking between the Golgi apparatus and ER, coverslips were scratched to enclose a small area and all the cells within that area microinjected. Cells were microinjected first with rab33b mutant at a plasmid concentration of 1000 ng/μl. After a 16 h expression period, cells were then either incubated with Cy3-Shiga-like toxin (SLT)-B-fragment as described [8] or microinjected a second time, with pSARA plasmid. Coverslip cultures were incubated then for an additional 2 or 6 h and fixed in formaldehyde. Protein distributions were quantified for an average of 10 cells per condition.

2.5. Immunofluorescence

Routinely, cells were fixed in 3.5% formaldehyde in PBS for 15 min, permeabilised and blocked in 0.1% saponin/0.2% gelatin in phosphate-buffered saline (SG/PBS) for 20 min and then incubated 1 h with the primary antibody. The cells were washed three times with SG/PBS and incubated for 20 min with the secondary antibody. Cells were again washed three times in PBS and mounted on microscope slides in Mowiol. For visualisation of TGN46, Mann II and VSV-G, cells were permeabilised in 0.1% Triton X-100 rather than saponin.

2.6. Bovine brain cytosol, HeLa extract and rat liver Golgi extract

Bovine brain cytosol was prepared as described [24]. For HeLa extract, S-HeLa cells were grown in spinner flasks. Cells were harvested and pelleted at 3000 rpm using a Sorvall GS-3 rotor for 10 min at 4°C. Cells were washed in PBS and pelleted twice. Cells were washed in 20 mM HEPES, 250 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.5 and re-pelleted. Cells were finally resuspended in extraction buffer (20 mM HEPES, 250 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, pH 7.5) and passed through a ball bearing homogeniser 20 times. The lysate was cleared by ultracentrifugation at 45 000 rpm for 60 min at 4°C using a TLA-45 rotor (Beckman). Extracts were then snap frozen in liquid nitrogen and stored at −80°C. Before incubation with GST fusion protein, the extract was centrifuged at 23 100×*g* for 30 min at 4°C. Typically the protein concentration was 25–30 mg/ml. Rat liver Golgi membranes and rat liver cytosol were prepared as described [38], snap frozen in liquid nitrogen and stored at −80°C. For Golgi extract, rat liver Golgi membranes were thawed and spun at 23 100×*g* for 15 min at 4°C. Pelleted membranes were resuspended in extraction buffer (as above) and then incubated at 4°C for 1 h rotating. Typically the protein concentration was 5 mg/ml. The extract was spun at 1000×*g* for 5 min at 4°C before incubating with GST fusion proteins.

2.7. GST-rab fusion protein chromatography and immunoprecipitations

GST-rab fusion proteins and GST were expressed in *Escherichia coli* and purified according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). GST-rab fusion proteins were used as baits to identify nucleotide-specific interacting components as described [24,39]. In short, GST-rab proteins stabilised in the GTP-bound or GDP-bound state were incubated with bovine brain cytosol, HeLa extract or rat liver Golgi extract. Interacting proteins were eluted as described [24,39]. Eluates were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and separated proteins were visualised by silver or Coomassie staining or transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) for Western blotting using the ECL method (Amersham Life Science, Amersham, UK) and X-Omat XAR5 film (Eastman Kodak Co., Rochester, NY, USA).

For immunoprecipitation anti-rabaptin-5 or anti-myc antibodies were coupled to protein G Dynabeads (Dynal, Hamburg, Germany), washed in PBS and incubated with HeLa extract for 1 h at 4°C rotating. The beads were washed three times with PBS containing 0.1% Triton X-100 and then incubated in SDS–PAGE Laemmli sample buffer for 15 min at room temperature. The beads were removed from the buffer and the sample subjected to SDS–PAGE followed by Western blotting.

2.8. Identification of interacting factors

Gel proteolytic cleavage was performed automatically in the 'Progest' as described [40,41] (Genomic Solutions Cambridge, UK). Peptides were analysed on a Bruker REFLEX matrix-assisted laser desorption ionisation time-of-flight mass spectrometer (Bruker Analytik, Bremen, Germany) equipped with a 337 nm N_2 laser and the grid-less delayed extraction option [42]. The peptide mass maps were recorded with mass resolution up to 15 000 and mass accuracy below 50 ppm. The products of trypsin autolysis were used as internal calibrants. A list of tryptic peptide masses generated from the mass spectrum was entered into the programme PeptideSearch [40] and used to screen a non-redundant sequence database (NRDB) maintained at the European Bioinformatics Institute in Cambridge, UK (ftp://ftp.ebi.ac.uk/pub/databases/PeptideSearch/).

3. Results

3.1. The Golgi-specific rab33b is involved in retrograde transport

Rab33b was originally identified in a general screen for small GTPases and was shown to localise to the *medial* Golgi apparatus at the ultrastructural level by immunoperoxidase labelling of KB cells (human pharyngeal carcinoma cells) [11]. Northern blotting revealed the expression of rab33b in most tissues and commonly used cell lines including HeLa cells. Indirect immunofluorescence revealed a compact juxtanuclear staining pattern in KB cells consistent with an exclusive Golgi localisation. To confirm Golgi localisation in HeLa

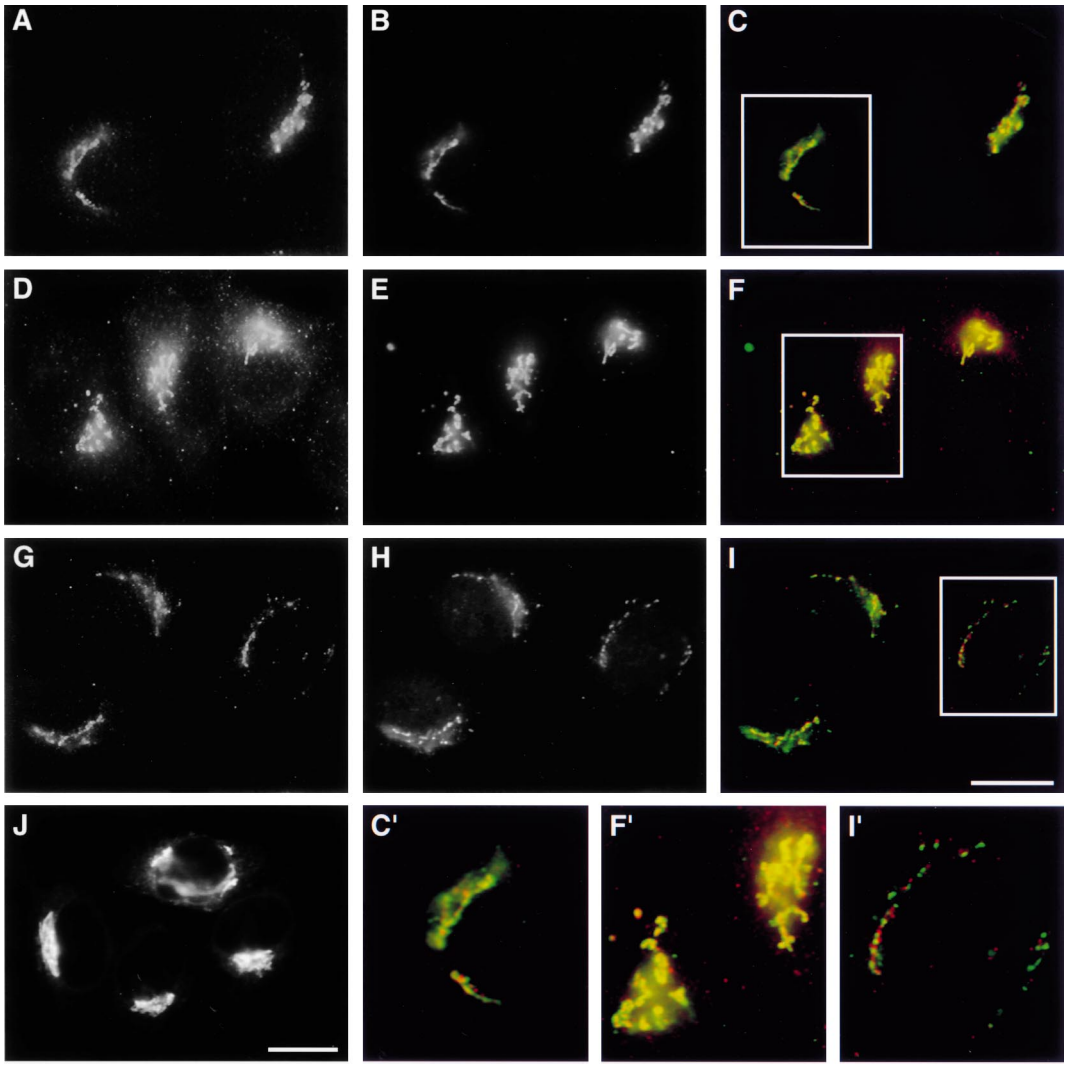

Fig. 1. Localisation comparisons of rab33b and Golgi markers. HeLa cells were fixed, permeabilised and stained for rab33b, gp27 (*cis*/CGN), Mann II-VSV (*medial*) and TGN46 (TGN). Note that rab33b does give the most extensive colocalisation with Mann II but less with gp27 and TGN46. A: Endogenous rab33b. B: Endogenous gp27. C: Overlay of rab33b and gp27. D: Endogenous rab33b. E: Mann II-VSV. F: Overlay of rab33b and Mann II-VSV. G: Endogenous rab33b. H: Endogenous TGN46. I: Overlay of rab33b and TGN46. J: YFP-rab33bwt stably expressed in HeLa cells. C′, F′ and I′: Higher magnification views of the boxed areas in C, F and I respectively. Scale bars represent 10 μm.

cells, used throughout this study, we stained for endogenous rab33b and compared this to the Golgi resident proteins, gp27(p24γ_3) (*cis* Golgi network/*cis*) [31], mannosidase II (Mann II) (*medial*/*trans*) [35] and TGN46 (*trans* Golgi network) [43]. As can be seen in Fig. 1, the stainings of endogenous rab33b (Fig. 1A) and gp27 (Fig. 1B) showed partial overlap (Fig. 1C) in the juxtanuclear area. Similarly, endogenous rab33b (Fig. 1G) revealed partial overlap (Fig. 1I) with endogenous TGN46 (Fig. 1H) whereas an almost complete colocalisation (Fig. 1F) was observed between endogenous rab33b (Fig. 1D) and Mann II (Fig. 1E) stably expressed in HeLa cells [35]. To rule out that rab33b was also localised outside the secretory pathway, a possibility raised by occasional scattered punctate structure as shown in Fig. 1D, G, we constructed a YFP-rab33b fusion protein and expressed this stably in HeLa cells. Pronounced juxtanuclear fluorescence for YFP-rab33b was observed in these cells with an additional faint perinuclear staining pattern consistent with an ER localisation of the rab protein (Fig. 1J). When overexpressed, rab33b had no tendency to accumulate in punctate, non-Golgi-like structures in the cytoplasm (Fig. 1J and also Fig. 3C, D). Golgi protein distribution was normal in the low expressing cells (Fig. 1J) as marked by GalT (data not shown).

As an initial test of the role of rab33b in the secretory pathway, we examined its effect on the anterograde transport of a temperature-sensitive mutant of the G protein of vesicular stomatitis virus (VSV) fused to GFP via its cytoplasmic domain, VSV-G_{tso45}-GFP [44]. Plasmid DNA encoding the VSV-G_{tso45}-GFP was microinjected into HeLa cells together with DNA encoding mutants of rab33b restricted in either the GDP (rab33b$_{T47N}$)- or the GTP (rab33b$_{Q92L}$)-bound state. Cells were then incubated at the restrictive temperature, 39.5°C, to accumulate the VSV-G_{tso45}-GFP in the ER for 6 h and then transferred to 31.5°C to release VSV-G_{tso45}-GFP from the ER. The total GFP fluorescence was compared to the cell surface staining of VSV-G_{tso45}-GFP using a monoclonal antibody (mAb) to the lumenal domain of VSV-G in cells fixed and incubated with mAb and red fluorescent secondary antibody before permeabilisation. Cells were then permeabi-

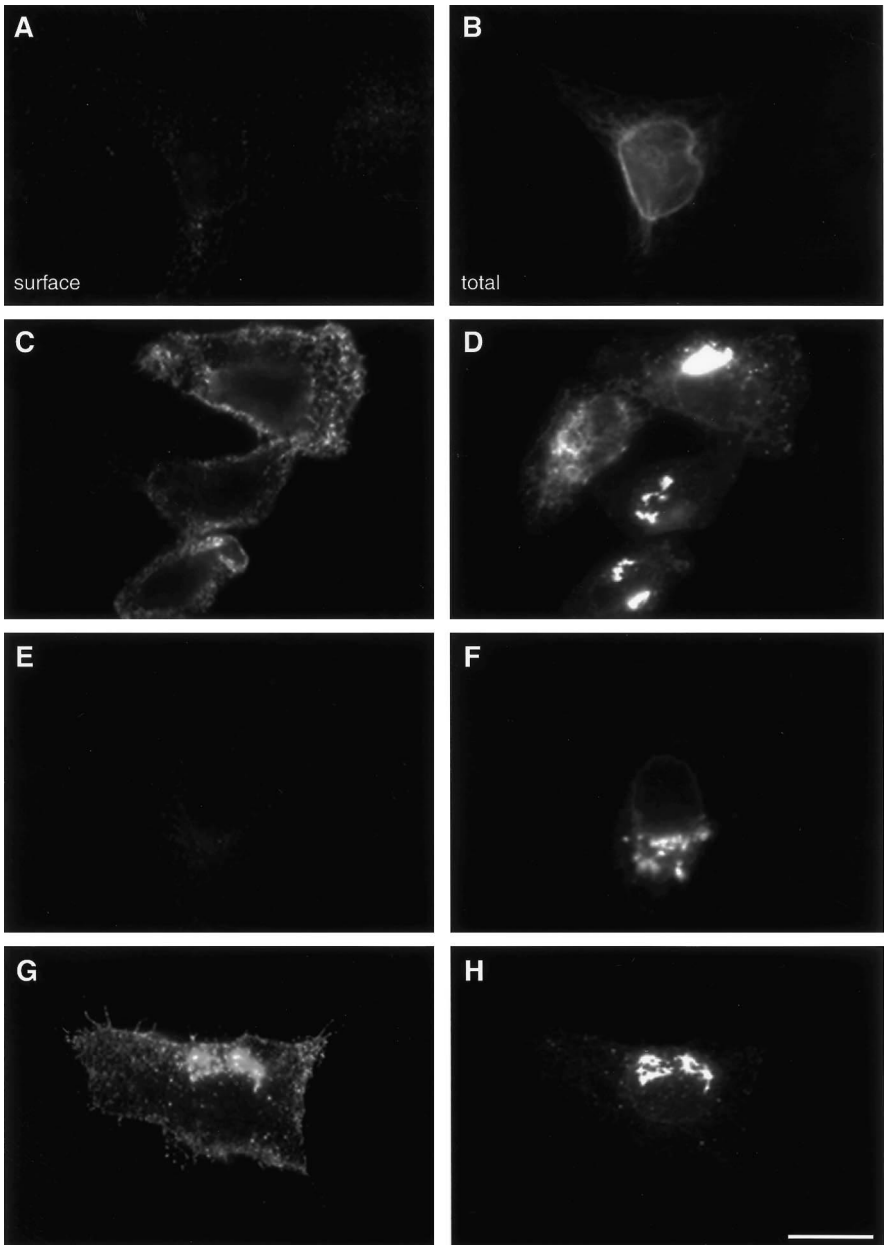

Fig. 2. GTP-bound rab33b inhibits VSV-G protein secretion. HeLa cells were microinjected with plasmid DNA encoding VSV-G_{tso45}-GFP, either alone or together with a plasmid DNA encoding the constitutively active mutant rab33b_{Q92L} or the inactive mutant rab33b_{T27N}. Cells were incubated at 39.5°C for 6 h and then chased for 1 h at 31.5°C before fixing. VSV-G transport to plasma membrane was examined by staining for the lumenal domain of VSV-G protein. A and B: Before chasing at 31.5°C VSV-G is ER localised (surface staining and GFP fluorescence, respectively). C and D: After 1 h chase VSV-G is found both in the Golgi and on the surface. E and F: Upon coinjection of rab33b_{Q92L}, VSV-G is transported to the Golgi but does not reach the plasma membrane whereas rab33b_{T47N} has little, if any, effect on the transport (G and H). I: Quantification analysis showed that little if any VSV-G is found on the cell surface when co-expressing rab33b_{Q92L}. Scale bar represents 10 μm.

lised for incubation with mAb directed against rab33b followed by blue fluorescent secondary antibody. This subsequent mAb incubation was required to identify the level of expression of the rab33b mutants. Only in cells exhibiting a high level of rab33b expression were phenotypic effects on secretion observed. Low level expression had no effect on VSV-G_{tso45} transport to the cell surface (data not shown).

Before transfer to permissive temperature VSV-G_{tso45}-GFP was exclusively seen in the ER and nuclear envelope, a characteristic extension of the ER (e.g. Fig. 2B), with little or no surface staining (Fig. 2A). Following 1 h at permissive temperature, only relatively small amounts of ER staining could be seen. Most of the VSV-G_{tso45}-GFP was seen instead in the juxtanuclear region of the Golgi apparatus (Fig. 2D) and at the cell surface (Fig. 2C and D). Upon high level expression of rab33b_{Q92L}, very little if any VSV-G_{tso45}-GFP could be detected at the cell surface (Fig. 2E). Instead, the main portion of the fusion protein appeared arrested in the juxtanuclear region of the cell, Golgi apparatus and/or intermediate compartment. In contrast, high level expression of rab33b_{T47N} had little if any visible effect on VSV-G_{tso45}-GFP transport to the cell surface (Fig. 2G and H). We presume that the concen-

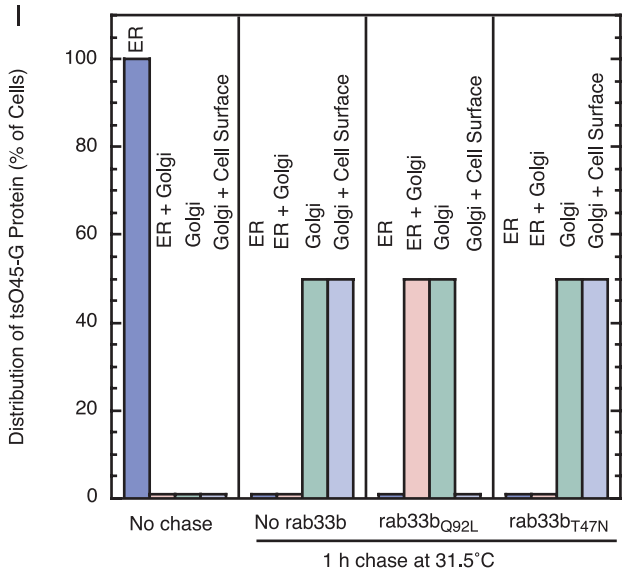

Fig. 2 (*Continued*).

trated fluorescence in the Fig. 2G cell surface image reflects either bleed through between fluorescence channels or antibody leakage from the cell surface staining procedure. The rab33b$_{Q92L}$-specific inhibition of VSV-G$_{tso45}$-GFP transport to the cell surface was confirmed through quantification when compared to either VSV-G$_{tso45}$-GFP alone or with rab33b$_{T47N}$ (see Fig. 2I). These data suggest that rab33b is needed for anterograde transport of VSV-G$_{tso45}$-GFP through the Golgi stack or subsequent downstream transport from the Golgi to the cell surface. This effect may be either direct or indirect. High level overexpression of the other known cisternal Golgi rab, rab6, blocks VSV-G$_{tso45}$ transport through the Golgi indirectly by disrupting the structural organisation of the Golgi apparatus; Golgi resident enzymes relocate to the ER [9].

To ask if rab33b also redistributed Golgi resident enzymes when overexpressed at the high levels required to inhibit VSV-G$_{tso45}$ transport to the cell surface, we tested the effect of these expression levels on the distribution of Golgi stack resident *N*-acetylgalactosaminyltransferase-2 fused to GFP (GalNAc-T2-GFP) [36]. HeLa cells stably expressing GalNAc-T2-GFP were microinjected with plasmid DNA encoding the different rab33b mutants or wild-type rab33b to achieve a high level of expression as assayed by antibody staining for rab33b. As can be seen in Fig. 3A, microinjection of rab33b$_{Q92L}$ caused ER accumulation as indicated here by the high level of cytoplas-

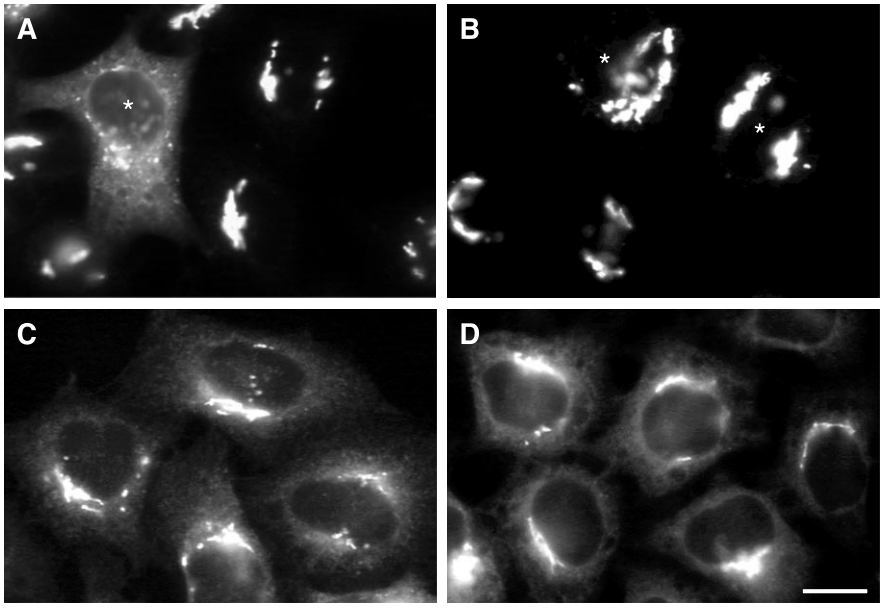

Fig. 3. Overexpression of GTP-bound rab33b relocalises Golgi resident enzymes to the ER. HeLa cells stably transfected with GalNAc-T2-GFP were microinjected with plasmids encoding the constitutively active mutant rab33b$_{Q92L}$ or the inactive mutant rab33b$_{T47N}$. After 6 h or 16 h incubation at 37°C cells were fixed, stained for rab33b, and the localisation of GalNAc-T2 examined. A: Cells overexpressing rab33b$_{Q92L}$ (asterisks) showed an ER localisation of GalNAc-T2. B: Rab33b$_{T47N}$ expressing cells (asterisks) still had a compact, juxtanuclear Golgi fluorescence even after 16 h. C and D: HeLa cells stably expressing YFP-rab33b mutants (rab33b$_{Q92L}$ and rab33b$_{T47N}$ respectively) show predominantly juxtanuclear localisation of the protein.

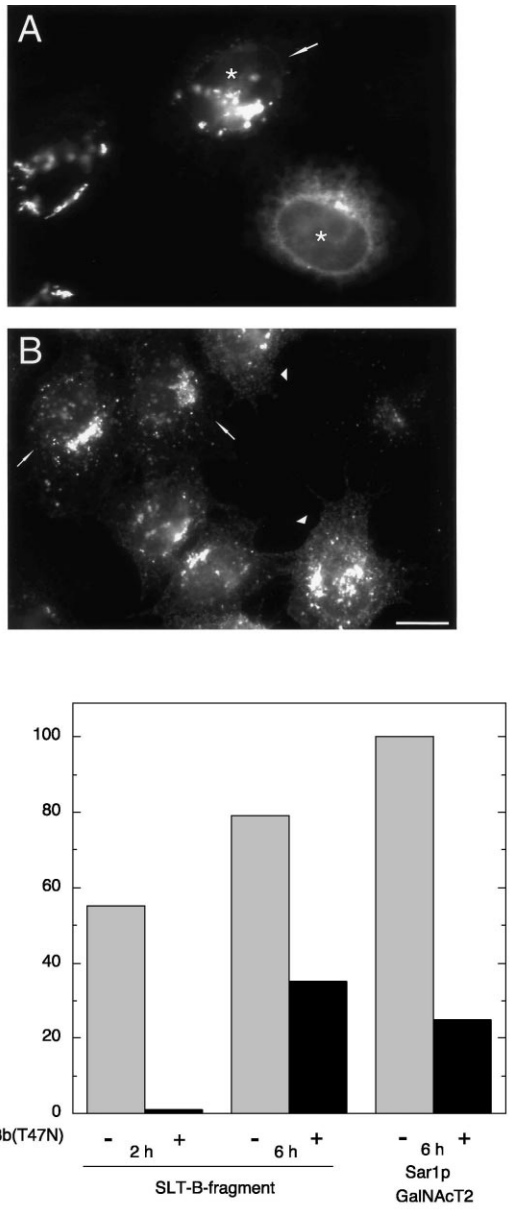

Fig. 4. GDP-bound rab33b inhibits Golgi to ER transport. A: HeLa cells stably expressing GalNAc-T2-GFP where microinjected with a plasmid encoding rab33b$_{T47N}$ mutant (arrow), incubated at 37°C for 16 h and then microinjected with a plasmid encoding the Sar1p^{dn} mutant in the presence of Cascade blue dextran coinjection marker (asterisks). Cells were incubated at 37°C for another 6 h and then fixed and stained for rab33b. As can be seen, Sar1p^{dn} inhibited ER export when injected alone and thereby caused accumulation of GalNAc-T2 in the ER. Upon co-expression with rab33b$_{T47N}$, Golgi to ER transport was blocked (arrow) as indicated by the lack of GalNAc-T2 relocalisation. B: HeLa cells were microinjected with a plasmid encoding rab33b$_{T47N}$ mutant (arrows) and incubated at 37°C for 16 h. Cells were then treated with Cy3-ST-B-fragment at 4°C for 30 min, cultured at 37°C for 6 h, and subsequently fixed and stained for rab33b. Transport of the B-fragment from Golgi to ER was blocked upon overexpression of rab33b$_{T47N}$ (arrow) whereas cells not expressing the mutant did show normal ER and Golgi localisation of the B-fragment (arrowheads). C: Quantification of the effect of rab33b$_{T47N}$ on transport of B-fragment or GalNAc-T2 to the ER. Control cells (−) or cells pre-expressing rab33b$_{T47N}$ (+) were either incubated with B-fragment at 4°C and then chased for 2 h or 6 h at 37°C, or microinjected with pSARA and incubated at 37°C for 6 h. Cells were fixed and stained for rab33b and distribution of B-fragment or GalNAc-T2 scored.

mic GalNAc-T2-GFP relative to non-injected cells whereas even with longer expression, 16 h versus 6 h, rab33b$_{T47N}$ did not (Fig. 3B). At sufficiently high expression, rab33b wild-type also produced ER accumulation of GalNAc-T2 (data not shown). Consistent with this, transient transfection to produce high level expression of either rab33b mutant into HeLa cells stably expressing Mann II revealed similar phenotypes (data not shown). Stable, low level expression of rab33b mutants fused to YFP in HeLa cells (Fig. 3C, D) did not cause any significant relocation of Golgi enzymes (data not shown) again indicating that high level expression is required to disrupt the normal cell phenotype. These data show that rab33b regulates Golgi organisation. Further they suggest that rab33b affects anterograde transport indirectly rather than directly. Comparative microinjection studies with rab6 mutants suggested that on a mass basis rab33b exerted less effect on Golgi to ER relocation than did rab6 (data not shown).

The GDP-bound mutant of rab6 selectively inhibits COPI-independent protein recycling between the Golgi apparatus and ER while having minimal effect on the juxtanuclear organisation of the Golgi apparatus [8]. Similarly, the GDP-bound mutant of rab33b (rab33b$_{T47N}$) had no effect on the distribution of Golgi resident enzymes. To test the effect of rab33b$_{T47N}$ on retrograde trafficking, we first expressed the mutant for 16 h in GalNAcT2-GFP HeLa cells and then introduced an ER exit block. As shown in Fig. 4A, the pSARA-encoded Sar1p^{dn} ER exit block (coinjection marker, Cascade blue dextran, not shown) produced an ER accumulation of GalNAc-T2-GFP in cells expressing Sar1p^{dn} alone (e.g. asterisk only). In cells expressing both Sar1p^{dn} (coinjection marker not shown) and rab33b$_{T47N}$ (antibody staining not shown) the GalNAc-T2-GFP distribution remained juxtanuclear (e.g. asterisk and arrow). In the alternative test, we incubated HeLa cells with Cy3-SLT-B-fragment, a known substrate of the COPI-independent Golgi recycling pathway [8,10]. As shown following a 6 h incubation, Cy3-SLT-B-fragment fluorescence accumulated at least partially over the cytoplasm in cells not expressing rab33b$_{T47N}$ (Fig. 4B, arrowheads) while in cells expressing rab33b$_{T47N}$ (antibody staining not shown) typically no cytoplasmic fluorescence was observed (Fig. 4B, arrows). Please note that although the extent of Cy3-SLT-B-fragment clearance from the Golgi apparatus in this experiment was low relative to that reported previously [8], the differences plus and minus rab33b$_{T47N}$ are nevertheless sufficient to indicate a rab-dependent effect. Results were quantified by scoring the effect of rab33b$_{T47N}$ either on the distribution of GalNAc-T2 in Sar1p^{dn} expressing cells or on the distribution of Cy3-SLT-B-fragment. In both cases, the quantification revealed that rab33b$_{T47N}$ inhibited Golgi to ER trafficking (Fig. 4C). We conclude that rab33b is a switch for retrograde trafficking between the ER and Golgi apparatus with rab33b$_{Q92L}$ and wild-type being in the positive state while rab33b$_{T47N}$ is in the negative state.

3.2. Identification of interacting components

Having established that rab33b is functionally significant with respect to the Golgi apparatus, we proceeded to identify molecular machinery components with which it interacted. Using the GST fusion protein approach established by Zerial and co-workers, we screened by two procedures for proteins, which interacted specifically with the GTP-bound form of rab33b but not with the GDP-bound form. In the first pro-

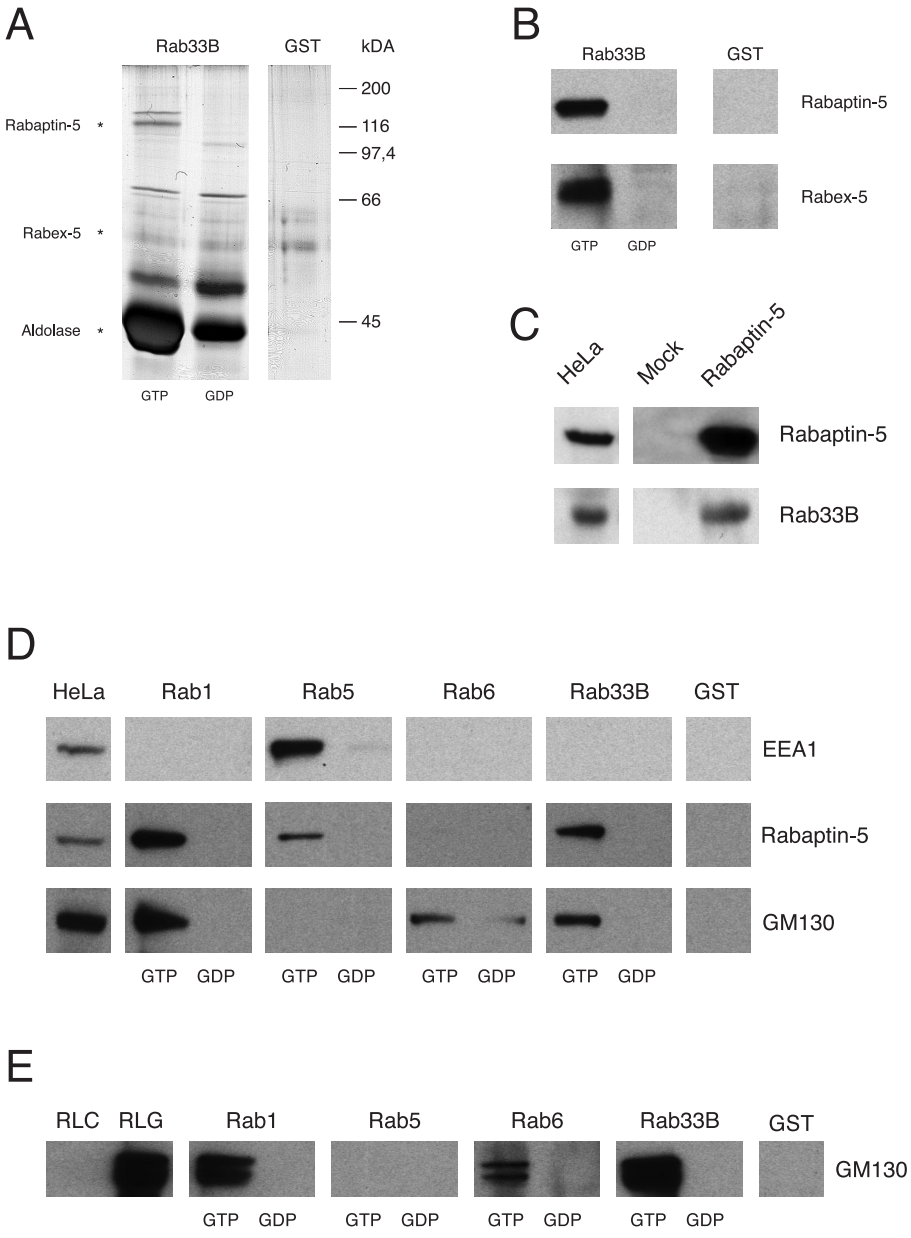

Fig. 5. Rab33b interacts with several factors in a GTP-specific manner. GST-rab33b columns stabilised in the GTP-bound or GDP-bound form were incubated with bovine brain cytosol, HeLa or rat liver Golgi extracts. A: Retained proteins from bovine brain cytosol were eluted from the columns, run on SDS–PAGE gels and silver stained. Several bands specific for the GTP-bound form of rab33b were sequenced and identified as rabaptin-5, rabex-5 and a brain-specific aldolase. B: Results from the sequencing were confirmed by Western blotting. Both bovine rabaptin-5 and rabex-5 interact specifically with the GTP-bound form of rab33b. C: Immunoprecipitation from HeLa extract using anti-rabaptin-5 antibodies brought down rab33b whereas the mock antibodies did not, as shown by Western blotting. D: HeLa extract was incubated with rab33b, rab1, rab5 and rab6. Retained proteins were eluted, run on SDS–PAGE for Western blotting and the membrane probed with different antibodies. EEA1 interacted specifically to the GTP-bound form of rab5 but to none of the Golgi rabs. Rabaptin-5 interacted specifically to the GTP-bound form of rab1, rab5 and rab33b and GM130 interacted with rab1, rab6 and rab33b but not with rab5. E: Rab columns were incubated with rat liver Golgi extract and eluted components run on SDS–PAGE for Western blotting and the membrane probed for GM130. Again, GM130 was found only on rab1, rab6 and rab33b columns but not on the rab5 columns.

cedure, components exhibiting specificity for interactions with two affinity columns, stabilised with GTPγS or with GDP, were identified by mass spectroscopy, and in the second by Western blotting. In initial mass spectrometry experiments, cytosols were prepared from bovine brain, and interacting components eluted with EDTA and separated by SDS–PAGE. Candidate components interacting specifically with the GTP-bound form of rab33b were then digested with trypsin, eluted from the gel and analysed by mass spectrometry. The most prominent band was that of a protein migrating at an apparent molecular weight of a 40 kDa. This protein was identified as a brain-specific aldolase. Of higher molecular weight proteins, two were identified as rabaptin-5 and rabex-5 (Fig. 5A). Note that whereas rabaptin-5 was readily visible by silver staining rabex-5 was not but could be faintly stained using Coomassie blue when loading a greater amount of proteins (data not shown). A total of 33 and 24 peptides were matched with high accuracy by mass spectrometry fin-

ger-printing to rabaptin-5 and rabex-5, respectively. That the higher molecular weight protein was indeed rabaptin-5 and not rabaptin-4 [45] was evident from one of the peptides in the mixture covering a region specific only for rabaptin-5 (data not shown). Rabaptin-5 and rabex-5 interaction with rab33b in its GTP-bound form was confirmed by Western blotting using specific antibodies (Fig. 5B).

The identification of aldolase, rabaptin-5 and rabex-5 as interacting components prompted us to examine if similar interacting proteins could be detected using other sources than bovine brain cytosol. Detergent extracts from HeLa cells were prepared and tested for interactions by an independent assay. Co-immunoprecipitation of rab33b and rabaptin-5 was performed using antibodies against rabaptin-5 (Fig. 5C). We then passed HeLa extracts over GST-rab33b columns as before. In those experiments we did not see a GTP-specific band at 40 kDa. For comparison, GTP- and GDP-stabilised columns of rab1, rab5 and rab6 were also examined. As shown in Fig. 5C, a GTP-specific interaction of the endosomal rab5 effector, EEA1, could only be seen with rab5. In contrast, rabaptin-5 was seen interacting specifically with rab1, rab5 and rab33b but not with rab6.

After having found that rabaptin-5 and rabex-5 interacted with Golgi rab proteins we decided to examine if peripheral proteins, implicated in the tethering of vesicles or stacking of Golgi membranes such as GM130, would also interact. As shown by Western blotting in Fig. 5D and E, GM130 interacted specifically with the GTP-bound form of rab33b, rab1 and rab6. GM130 was not retained on rab5 columns. That EEA1 but not GM130 was shown interacting with the GTP-bound form of rab5 suggests that indeed, the observed interactions were of a highly specific nature. To further support this conclusion, we assessed the effectiveness of the GTPγS procedure in producing nucleotide exchange from GDP to GTP to generate a fully GTP-bound rab column. This is important because the presence of GDP-bound rab reduces column capacity for binding GTP state-specific rab effectors. Our GDP rab probe was rab GDP dissociation inhibitor, rab-GDI. Rab-GDI is a general rab effector that binds to the GDP- not GTP-bound form of rabs [46]. The binding of rab-GDI to each column was then detected by Western blotting. No rab-GDI could be detected in the eluant from the GTP-stabilised columns of rab33b or rab5 but only in the eluant from the respective GDP-stabilised columns. On the other hand some rab-GDI was seen binding to the GTP-stabilised columns of rab1 and rab6 suggesting that these two rab proteins had not exchanged from GDP to GTP as well as rab5 and rab33b (data not shown). Hence the results from the rab1 and rab6 GTP-stabilised columns might have underestimated nucleotide-specific binding of interacting components.

4. Discussion

Our experiments had two goals. First, to establish that rab33b, a novel Golgi-associated rab localised to *medial* cisternae was of functional significance with respect to the organelle. Second, to ask if rab33b was associated with a unique or overlapping set of interacting proteins, i.e. putative effector molecules. The current consensus is that each rab protein has a relatively specific set of effectors. We report that rab33b is involved in retrograde transport between the Golgi apparatus and ER. Likely its effect on anterograde transport to the cell surface is an indirect consequence of its involvement in retrograde transport. The regulatory effect of rab33b seems to occur through an overlapping, combinatorial set of protein interactions. We find that rabaptin-5, rabex-5, GM130 interacted specifically with the GTP-bound form of rab33b. These in vitro interactions overlapped at least in part. We found GM130, rabaptin-5, rabex-5 interacted with rab1, involved in ER to Golgi transport, GM130 with rab6, localised to *medial* to *trans* cisterna, and rabaptin-5 and rabex-5 with rab5, the endosomal rab from which rabaptin-5 and rabex-5 derive their names. The in vitro discovery of novel rabaptin-5 and rabex-5 interactions clearly validates our initial choice of the relatively unbiased procedure of mass spectroscopy identification of interacting components versus Western blotting. Our in vitro observations challenge the current dogma of how rabs function and clearly indicate the need for further mass spectroscopic probing of rab interactions.

Rab33b was found to partially colocalise with the CGN/*cis* marker gp27 and the TGN marker TGN46 but the best overlap was with the *medial* Golgi marker Mann II. This is consistent with previously published data [11]. Overexpression of the constitutively active mutant of rab33b caused the relocation of Golgi resident enzymes to the ER. The inactive mutant prevented or slowed down the recycling. This is shown by the fact that expression of the $rab33b_{T47N}$ mutant blocks GalNAc-T2 in the Golgi in majority of cells when ER export is inhibited using $Sar1p^{dn}$. A similar block was seen for retrograde movement of SLT-B-fragment upon overexpression of $rab33b_{T47N}$. These data strongly suggest that rab33b has a regulatory role in retrograde transport and provide an explanation for the inhibition of VSV-G protein transport from the ER to the cell surface. Similar results were obtained by Girod et al. [8] using $rab6_{T27N}$ although rab33b seems to have somewhat less drastic effects. Longer expression times were needed for the relocation of Golgi enzymes to the ER using the constitutively active mutant of rab33b. Conversely, a complete inhibition of Golgi to ER transport was not observed upon expression of $rab33b_{T47N}$. A possible interpretation of these results is that rab33b acts upstream (*medial*) of rab6 (*trans*) in regulating Golgi to ER COPI-independent recycling. As suggested previously [47], a likely route for Golgi to ER recycling is through the direct but regulated fusion between cisternal remnants and the ER at the *trans* side of the Golgi stack. This is made possible by the juxtaposition of these two compartments [48]. Indeed, components of the *trans* cisternae appear to redistribute faster than those of the *medial* cisternae making this scenario a likely one [49].

Having established a functional importance of rab33b, we then 'fished' for interacting proteins. Using a GST fusion protein approach we found an interesting set of components that interacted with rab33b in a GTP-specific manner. Out of those, three proteins were sequenced as rabaptin-5, rabex-5 and a brain-specific aldolase by mass spectrometry. Rabaptin-5 and rabex-5 have so far been implicated as specific effectors in the endocytic pathway, being necessary for fusion of early endosomes [25,26,29]. It was therefore a surprise to find them interacting with rab33b, which shows little if any tendency to localise to endosomes. Moreover, this suggested to us that rab33b, rabaptin-5 and rabex-5 might be involved in the docking and fusion of vesicles in the Golgi apparatus or in homotypic fusion events between Golgi membranes/cisternae. We then probed, by Western blotting, for interacting Golgi

proteins in HeLa and rat liver Golgi extracts and found that the tethering/stacking protein GM130 was also retained on the GST-rab33b fusion protein columns in a GTP-specific manner. Preliminary experiments also suggest that GRASP65 interacts with rab33b (work in progress). Upon Western blotting comparisons with other Golgi localised rab proteins as well as with the endosomal rab5, we found that rab1 bound essentially the same factors as rab33b and neither bound EEA1, an endosomal protein known to interact with rab5 [24]. Recently, Weide et al. [14] and Moyer et al. [13] both identified GM130 as an effector of rab1 and in addition Moyer et al. have shown that GRASP65 interacts with rab1. Contrary to our data, Weide et al. failed to find an interaction between GM130 and rab6. We found that the interaction of GM130 with the GTPγS-stabilised rab6 column was weak. Most likely this was due to a relatively poor nucleotide exchange in vitro. Such a technical limitation may also have affected the data of Weide et al. We would like to suggest that GM130, rabaptin-5 and rabex-5 are all effectors of rab33b as well as rab1. This raises the interesting possibility that rab proteins have overlapping combinatorial effector sets. Balch and colleagues [13] have proposed on the basis of in vitro assays with rab1 that the same rab protein can interact with different effectors at donor and acceptor membranes. Our findings increase the range of combinations to be considered between rab proteins and effector molecules.

In conclusion, we show that rab33b has a role in regulating retrograde traffic between the Golgi apparatus and ER. We find that rab33b interacts in a nucleotide-specific manner with a putative effector set that includes overlapping components used by rab1 and the endosomal rab5. Our work also illustrates the need for unbiased identification procedures such as systematic mass spectroscopy analysis if we are to know the full set of rab protein interactions.

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