A role for the small GTPase Rab21 in the early endocytic pathway

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Summary

Rab proteins comprise a family of monomeric GTPases that control cellular membrane traffic. Rab21 is a poorly characterised member with no known function. Human Rab21 cDNA from K562 cells was subcloned into GFP expression vectors to generate Rab21 and Rab21 mutants defective in either GTP hydrolysis (Rab21 Q78L) or binding (Rab21 T33N) for transfection studies in HeLa cells. Confocal fluorescence microscopy and ultrastructural studies revealed Rab21 to be predominantly localised to the early endocytic pathway, on vesicles containing early-endosomal antigen 1 EEA1, transferrin receptor and internalised ligands. EEA1 was localised to enlarged endosomes in Rab21 wild-type expressing cells but the GTP hydrolysis and GDP binding mutants had unique phenotypes labelling tubular reticular structures and the trans-Golgi network, respectively. Early endosome localisation for Rab21 was confirmed in a hepatoma cell line that allowed analysis of the subcellular distribution of the endogenous protein. Comparison of the localisation of Rab21 with other Rabs revealed extensive colocalisation with early endocytic variants Rab4, Rab5, Rab17 and Rab22 but much less overlap with those associated with late endosomes, recycling endosomes and the early secretory pathway. Cells expressing Rab21 T33N had defects in endocytosis of transferrin and epidermal growth factor and failed to effectively deliver the latter ligand to late endosomes and lysosomes for degradation. Collectively, our data provide the first characterisation of Rab21 function in early endosome dynamics.

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Key words: Endosomes, Endocytosis, Rab proteins, Epidermal growth factor, Transferrin

Introduction

Cellular membrane traffic orchestrates the directed transport of material in membrane-bound compartments along defined pathways. These events are controlled by specific interactions of membrane trafficking proteins with each other, with their cargo and with organelle lipids. Mammalian Rab proteins comprise a family of approximately 60 monomeric GTPases that are involved in membrane trafficking processes within cells (Pfeffer, 2001; Segev, 2001; Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). Rabs interact with a multitude of effector and effector proteins to orchestrate, via their GTPase activity, multiple roles in membrane traffic, including membrane fusion, organelle biogenesis and directed transport of organelles along cytoskeletal frameworks. Well-characterised members include Rab1 and Rab2 in the biosynthetic pathway and Rab5 and Rab7 in the degradative endocytic pathway.

At least 12 members of the Rab family have now been implicated in controlling the dynamics of endocytic pathways (Segev, 2001; Somsel Rodman and Wandinger-Ness, 2000; Stenmark and Olkkonen, 2001). By far the best characterised of these is Rab5, which, in collaboration with an expanding variety of binding partners, operates on early endosomes and the plasma membrane to control early endocytic traffic and signalling (Lanzetti et al., 2004; Miaczynska et al., 2004; Zerial and McBride, 2001). Other less characterised variants include Rab4 (Daro et al., 1996) and Rab11 (Chen et al., 1998; Duman et al., 1999; Ren et al., 1998; Ullrich et al., 1996), which operate distally to Rab5 on recycling pathways, and Rab7, which functions in late endocytic traffic (Feng et al., 1995; Gorvel et al., 1991). Colocalisation of endocytic Rabs 4, 5 and 11 with each other and with internalised cargo at different endocytic time points suggests that combinations of Rabs may share a single endosome and that Rab domains on endosomes define the localities of internalised molecules as they traverse through the endocytic pathway (Sonischens et al., 2000; Zerial and McBride, 2001). However, several other putative endocytic Rab variants await characterisation, and as there is little current consensus for a generalised mechanism of Rab function on any pathway, it is important to determine location and functional roles for these undefined members.

One such variant is Rab21, which was originally cloned and...
sequenced from the canine MDCKII cell library (Zerial and Huber, 1995), and more recently from human intestinal epithelial CaCo-2 cells (Opdam et al., 2000). Distribution of Rab21 in CaCo-2 cells was dependent on cell polarity, with an endoplasmic reticulum-like localisation suggested for nonpolar cells and an apical vesicular distribution in polarised cells and human jejunal tissue (Opdam et al., 2000). Although a specialised function in epithelial cells is commonly reported from the localisation studies in CaCo-2 cells, the study also showed that Rab21 was ubiquitously expressed and no functional Rab21 data have been reported in any cell line. Rab21 is more closely homologous to endocytic Rab5 and Rab22 than other Rabs, and phylogenetic analysis co-segregated these three variants into a single functional group (Pereira-Leal and Seabra, 2001). This group contained human Rab5 and Rab22 isoforms, and Rab5 and Rab21 variants from Caenorhabditis elegans and Drosophila melanogaster genomes; these organisms do not contain Rab22 homologues (Pereira-Leal and Seabra, 2001). Human Rab22a has been localised to early and recycling endosomes and loss of function or expression of Rab22a mutants influence several traffic steps at these locations (Kauppi et al., 2002; Mesa et al., 2001; Weigert et al., 2004).

We cloned Rab21 from a K562 cDNA library and in this study we show, using subcellular localisation studies and functional assays, that Rab21 is predominantly localised to the early endocytic pathway, and that expression of Rab21 variants affect endosome morphology and function.

Materials and Methods

Reagents and antibodies

Antibodies against the early-endosomal antigen EEA1 (N-19) were from Insight Biotechnology (Wembley, UK), anti-Lamp-1 (H4A3) antibodies were from DSHB, (Iowa, USA), anti-p230 antibodies were from BD Biosciences (Heidelberg, Germany) and anti-green fluorescent protein (GFP) antibodies for immunoprecipitation/enhanced chemiluminescence (ECL) were from Roche (Mannheim, Germany). Anti-epidermal growth factor receptor (EGFR) antibodies were from Neomarkers (Soham, UK), anti-transferrin receptor antibodies (H68.4) were from Zytomed GmbH, (Berlin, Germany) and anti-Rab21 antibodies were as described previously (Opdam et al., 2000). Anti-EEA1 antibodies [used for labelling thawed cryosections (Mills et al., 1998)] and anti-Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) antibodies (Sachse et al., 2002) were a gift from Michael Clague (University of Liverpool, UK). Secondary antibodies for immunofluorescence microscopy, tetramethylrhodamine-epidermal growth factor (EGF), Texas Red human transferrin and polyclonal anti-GFP antibodies for labelling thawed cryosections were from Molecular Probes (Leiden, Netherlands). Peroxidase-conjugated anti-mouse antibodies and 32P-GTP were from Amersham Biosciences. Human transferrin (Tf) was from Sigma (Gillingham, UK).

DNA constructs

The isolation of clones containing the GTP binding motifs of Ras-like GTPases from the K562 cell library was previously described (Green et al., 1997). Database searches confirmed six clones designated RabK562 to be fragments of human Rab21 (GenBank accession number AF091035) (Opdam et al., 2000). RT-PCR was performed using gene-specific primers to generate a template for subsequent nested PCR. DNA sequence analysis of this product confirmed the sequence previously published for human Rab21 (Opdam et al., 2000). The product of the nested amplification was then digested with BamHI and XbaI for directional subcloning into the pEFG-lag vector (Luo et al., 1996). The resulting vector encoded Rab21 with its first N-terminal amino acid residues MAAGGGGGAAMAA missing. Full-length Rab21 constructs were therefore generated by a final PCR amplification using these expression plasmids as templates, and an oligonucleotide encoding the missing N-terminal residues. The resulting full-length open reading frames were cloned into the pEGFP-C1 and pEYFP-C1 vectors (Clontech) using BglII and HindIII sites. All clones were verified by sequencing. Primers were constructed to generate two point mutants, Rab21 Q78L and Rab21 T33N, using the QuickChange PCR method (Stratagene). The primers employed were: for Rab21 Q78L, 5′-GGGAGATCAGGAGCTTCAAGAGAGATTCCAT and 5′-ATGGAATCTCTCTAGACCTGCCGGTGGTGCTG and 5′-TGGGATACGGCAGGTCTAATCCCA and for Rab21 T33N, 5′-TGGGATACGGCAGGTCTAATCCCA and for Rab21 T33N, 5′-TGGGATACGGCAGGTCTAATCCCA and for Rab21 T33N, 5′-TGGGATACGGCAGGTCTAATCCCA and for Rab21 T33N, 5′-TGGGATACGGCAGGTCTAATCCCA.

DNA sequence analysis confirmed these nucleotide changes in the resulting plasmids.

Human cDNA clones containing the open reading frames of other Rab proteins were identified by the IMAGE Consortium (LLNL, Livermore, USA), and were obtained from the RZPD (Berlin, Germany). Specifically, these were Rab1a (BC000905/IMAGE-E:2900705), Rab4a (BC002438/IMAGE:3346455), Rab5a (BC018288/IMAGE:345268), Rab7 (BC008721/IMAGE:2821435), Rab9a (BC017265/IMAGE:4139714), Rab11a (BC013348/IMAGE:351033-9), Rab17 (BC050426/IMAGE:5761178) and Rab22a (BC015710/IMAGE:3907891). Each was cloned by PCR into the pECFP-C1 vector (Clontech), and sequence verified.

Cell culture

HeLa cells (CCL-2) were grown in α-MEM medium, with Glutamax-I, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C with 5% CO₂. Human hepatocellular carcinoma Hep3B cells (HB-8064) were grown in DMEM medium with Glutamax-I, supplemented with 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin. All reagents were from Invitrogen.

Immunoprecipitation and GTP binding assays

1.8×10⁶ HeLa cells on 15 cm dishes were transfected with either the GFP-tagged Rab21 constructs, or with the empty GFP vector using FuGENE6 (Roche) according to the manufacturer’s instructions. After 20 hours the cells were washed three times with cold PBS then lysed with IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P40). One milligram from each sample was precleared for 2 hours followed by immunoprecipitation overnight using rabbit anti-GFP antibodies. Immunoprecipitates were captured with protein A Sepharose (Amersham Biosciences), washed five times with IP wash buffer (PBS, 0.1% Nonidet P40) and subjected to SDS-PAGE followed by western blotting to polyvinylidene fluoride (PVDF) membranes. Membranes were washed at room temperature for 1 hour in GTP-binding binding buffer, then for a further 1 hour in fresh buffer containing 1 μCi/ml 32P-GTP as previously described (Jones et al., 1999). After four washes with GTP-binding buffer, membranes were dried and 32P-GTP was analysed and quantified by Phosphorimaging with a FUJIFILM FLA.200 Image Reader. GFP or GFP-tagged Rab21 proteins were detected on these same membranes using mouse anti-GFP antibodies, followed by peroxidase-conjugated anti-mouse secondary antibodies and then ECL detection. Quantification was made using Scion Image software (NIH, USA).

Calcium phosphate transfection

1×10⁶ cells were seeded on glass coverslips in 35 mm wells and incubated for 14-16 hours before exchanging the medium with fresh...
complete medium 1 hour before transfection. For transfection in six-well plates, 37 µl 2.5 M CaCl₂ was added to 438 µl water and 12.2 µl of 0.5 µg/µl plasmid. This solution was slowly dropped onto 370 µl bubbled 2X HBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.1) and left to precipitate for 30 minutes at room temperature. Medium was removed from the wells and 123 µl DNA precipitate was added directly onto the cells in each well. Cells were incubated for 30 minutes at 37°C before the addition of 1.23 ml of complete medium. Cells were incubated for 8-9 hours before replacing media with α-MEM containing 15% v/v glycerol and incubating for 2 minutes at 37°C. The cells were washed three times in α-MEM and twice in complete medium and incubated for 25-30 hours before fixing or performing further experimental procedures.

Immunolabelling and fluorescence microscopy
Following transfection with calcium phosphate as above, or at the end of each experiment, cells on coverslips were washed in PBS and fixed for 15 minutes in 3% v/v paraformaldehyde in PBS. Cells were washed three times in PBS, quenched with 50 mM NH₄Cl/PBS for 10 minutes and permeabilised with a 5 minute incubation in 0.2% Triton X-100/PBS. Nonspecific binding sites were blocked by a 30 minute incubation in blocking buffer (2% v/v FCS, 2% w/v BSA in PBS). Primary and secondary antibodies were diluted in blocking buffer and incubation in blocking buffer (2% v/v FCS, 2% w/v BSA in PBS). Fluorescent images were obtained on a Leica DMIRB inverted microscope equipped with 40× oil-immersion objectives, and using standard Leica software.

Cryosectioning and immunogold labelling
Cells transfected with GFP-Rab21 wt plasmid, using calcium phosphate, were prepared in 60 mm tissue culture plates as described with the exception that they were maintained in complete medium for 48 hours after removing the precipitate. The cells were fixed in complete medium by addition of an equal volume of double-strength fixing solution (0.4 M phosphate buffer pH 7.4 containing 4% v/v PFA, 0.5% v/v glutaraldehyde). Cells were incubated at room temperature for 10 minutes before overnight incubation at 4°C in single strength fixing solution. Preparation of material for cryosectioning and immunolabelling was then performed as previously described (Griffiths, 1993; Slot and Geuze, 1983). Double labelling was carried out by the sequential labelling procedure of Slot et al. (Slot et al., 1991). Where mouse primary antibodies were used, a sandwich, rabbit anti-mouse polyclonal antibody was used before the Protein A gold step.

Texas Red transferrin uptake and chase into recycling compartments
Hep3B or HeLa cells were plated on coverslips in 6-well plates and the next day transfected with FuGENE6 according to the manufacturer’s instructions. Twenty-four hours post transfection the cells were depleted of bovine transferrin by incubation for 45 minutes in SFM-HEPES containing 0.2% BSA. The medium was removed and replaced with the same medium containing 50 nM Texas Red transferrin. The cells were incubated at 16°C or 37°C for designated time periods before washing three times for 1 minute on ice with PBS containing 1 mM CaCl₂, 1 mM MgCl₂, 0.2% BSA and once with PBS. The cells were either fixed and processed for fluorescence microscopy or incubated further for 30 minutes at 37°C in complete medium containing 1 µM human transferrin. The cells were then washed on ice and processed for fluorescence as described.

Epidermal growth factor endocytosis, degradation and quantification
GFP-Rab21 transfected cells were washed with serum-free medium containing 25 mM Na₂-HEPES pH 7.4 (SFM-HEPES) containing 0.2% w/v BSA and incubated for 30 minutes at 37°C with 75-150 ng/ml tetramethylrhodamine-conjugated EGF (Rh-EGF) in SFM-HEPES 0.2% BSA. The cells were placed on ice and washed three times for 1 minute in ice-cold PBS containing 0.5% BSA and once in ice-cold PBS, fixed and analysed by confocal fluorescence microscopy directly or after immunolabelling with antibodies against Lamp-1. For quantification of Rh-EGF, cells were incubated with Rh-EGF as above for 15 and 30 minutes. Cells were then selected based on the expression of moderate levels of GFP-Rab21 variants. Fluorescent images were acquired using constant exposure times and quantification of EGF uptake was performed using ImageJ software (NIH, USA). Areas of interest (70×70 pixels, corresponding to 10×10 µm) in both transfected and nontransfected cells were randomly selected. After thresholding, the number of distinct structures were counted, and the area and mean fluorescence for each of these structures was then measured. From these measurements the total fluorescence for the thresholded structures was calculated. Final results were based on the mean values obtained from ten cells for each construct transfected and at each time point after EGF addition. Results are expressed as mean values and standard deviations from the mean. Statistical differences were calculated using Student’s t-test for unpaired data.

For measuring EGF degradation, cells transfected with GFP-Rab21 constructs were incubated with Rh-EGF as above for 1 hour at 37°C. The cells were washed three times for 2 minutes in SFM-HEPES 0.5% BSA and further incubated at 37°C (chase) in SFM-HEPES 0.2% BSA for 2 hours. The cells were washed three times in ice-cold SFM-HEPES 0.5% BSA and once in PBS before fixing, permeabilising and processing for fluorescence microscopy. All fluorescent images were acquired using constant exposure times.

Live cell imaging
Cells grown on glass-bottomed dishes were transfected with GFP-Rab21 constructs using calcium phosphate as described above. Immediately before the experiment, medium was replaced with DMEM (without Phenol Red) supplemented with 25 mM HEPES and 0.5 g/l bicarbonate, pH 7.4. Cells were maintained at 37°C and imaged at 1.0 frame per second using an UltraView real-time confocal system (Perkin Elmer) consisting of a Nikon Eclipse TE200 microscope equipped with a 1.3 NA PlanFluar 100× objective. Images were stacked using NIH ImageJ software and processed as QuickTime files.

Results
Subcellular localisation of GFP-Rab21
Rab21 was identified from a K562 cDNA library (Green et al., 1997), and subsequently cloned and confirmed to be the same as that previously sequenced from a human CaCo-2 cDNA library (Opdam et al., 2000). This latter study described Rab21 to be localised at the endoplasmic reticulum and endosomes in nonpolarised and polarised cells, respectively (Opdam et al.,
The detection of immunoprecipitated proteins revealed a similar comparative expression levels and ability to bind GTP. ECL anti-GFP antibodies. This allowed a measurement of their immunoisolated the expressed proteins from cell lysates using blotting, GTP binding assays, fluorescence and electron microscopy. We initially transfected cells with empty GFP constructs or those encoding Rab21 variants and immunoprecipitated the expressed proteins from cell lysates using anti-GFP antibodies. This allowed a measurement of their comparative expression levels and ability to bind GTP. ECL detection of immunoprecipitated proteins revealed a similar level of expression of the three Rab21 variants in HeLa cells with the chimaeric proteins migrating at the expected molecular weight of 51 kDa, in contrast to GFP alone, which migrated as a 27 kDa band (Fig. 1A). The GTP binding abilities of these proteins were then determined by incubating the same membranes in the presence of 32P-GTP, and subsequent quantification by phosphorimaging. Despite observing similar levels of immunoprecipitated proteins we found that Rab21 Q78L consistently bound higher levels of GTP compared with the wild-type protein (Fig. 1B). By contrast, Rab21 T33N showed minimal ability to bind the nucleotide, and no binding by GFP alone was detectable (Fig. 1A-C).

Subcellular distribution of Rab21 variants was then analysed in transiently transfected HeLa cells using fluorescence microscopy. Despite lower transfection efficiencies using calcium phosphate (10-40%), this method was preferred in HeLa cells over lipid and viral based protocols as we observed both more consistent and generally lower expression levels; this allowed more precise analysis of Rab21 localisation. The single residue mutations had significant effects on Rab21 distribution, and cells that we have identified as expressing moderate levels of GFP-Rab21 are represented in Fig. 1D-F. GFP-Rab21 wt was concentrated in the perinuclear region of the cell with additional labelling of punctate structures emanating from these regions, towards and adjacent to the cell periphery (Fig. 1D). The subcellular distribution of the protein was identical in cells transfected with a haemaglutinin-tagged Rab construct (not shown), thus making it unlikely that GFP was affecting Rab21 localisation. Rab21 T33N was concentrated in a more compact perinuclear pattern in addition to a higher degree of diffuse cytoplasmic labelling, as expected for Rab mutants defective in GTP binding (Fig. 1E). However, very few punctate structures containing Rab21 T33N were visible. Rab21 Q78L was also located in perinuclear regions and more peripheral structures; however, we were surprised to observe that this protein was also localised to a reticular tubular network that emanated from the perinuclear region (Fig. 1F). These structures can be more clearly visualised in the accompanying movies of GFP-Rab21 expressing cells (see supplementary material, Movies I-3).

We then used a range of antibodies against organelle marker proteins to further identify the nature of the Rab21-positive structures. Owing to the observed perinuclear localisation of Rab21 wt, we initially utilised antibodies recognising proteins of the Golgi apparatus. Immunolabelling for the trans-Golgi network (TGN) marker p230 (Erlich et al., 1996) revealed co-localisation with GFP-Rab21 in the perinuclear area, but more peripheral red and green signals were generally distinct (Fig. 2A-C, arrowheads). Immunolabelling of the TGN with p230 showed that a large fraction of Rab21 T33N, despite its inability to efficiently bind GTP, is localised to this Golgi region (Fig. 2D-F). Expression of this variant did not affect p230 distribution. To identify the nature of the punctate Rab21 structures, we next labelled cells with markers of endosomal/lysosomal compartments. There was negligible co-localisation in the perinuclear region between Rab21 and Lamp-1, a marker of late endosomes and lysosomes, and no Lamp-1 labelling was detected on the large peripheral Rab21 structures (Fig. 2G-I, arrowheads). The transferrin receptor (TfR) localises to the plasma membrane, and early and recycling endosomes, and a significant degree of co-localisation...
of this protein with Rab21 was observed in punctate structures throughout the cell (Fig. 2J-L, arrows). There were, however, several distinct Rab21 and TIR structures (Fig. 2J-L, arrowheads). To further confirm the presence of Rab21 on endocytic membranes, cells expressing Rab21 wt were incubated with rhodamine (Rh)-labelled epidermal growth factor (EGF). Plasma membrane binding of EGF to the EGF receptor (EGFR) leads to the rapid internalisation of ligand-receptor complex and delivery through the endosomal pathway to the lysosome (Sorkin, 2001). EGF was internalised for short periods of time and the cells fixed. Partial colocalisation of GFP-Rab21 and Rh-EGF was apparent after only 6 minutes of internalisation (Fig. 2M-O), but the degree of colocalisation did not significantly increase after longer incubation times of 15 and 30 minutes (not shown). At the light microscope level, we did not observe any major effects of expression of any of these Rab21 variants on the distribution or morphology of organelles marked by antibodies in Fig. 2.

To gain further insight into exactly which endocytic structures are labelled by Rab21 wt, we performed colocalisation studies with other human Rab proteins whose localisation and characterisation is more established. We created expression constructs encoding seven different cyan fluorescent protein (CFP)-tagged wild-type Rab proteins, all known to localise to various endocytic organelles, and also CFP-tagged Rab1a, a protein well characterised in the early biosynthetic pathway (Plutner et al., 1991). These were cotransfected with yellow fluorescent protein (YFP)-tagged wild-type Rab21 into HeLa cells and visualised by confocal microscopy. Most strikingly we observed a very high degree of colocalisation with Rab5a (Fig. 3A-C), with greater than 90% of the Rab21-positive structures also containing this endocytic Rab (Fig. 3G). A similarly high degree of
colocalisation was also observed with Rab4a, Rab17 and Rab22a (Fig. 3G). However, distinctly lower levels of colocalisation were observed with Rab proteins known to operate in later endocytic compartments, such as Rabs 7, 9a and 11a. For example, with Rab11a, a protein known to function on recycling endosomes (Somsel Rodman and Wandinger-Ness, 2000), only 28% of Rab21 structures colocalised with this Rab (Fig. 3D-F,G). The specificity of such localisation analysis was further highlighted by the observation that less than 10% of the Rab21 structures overlapped with Rab1a (Fig. 3G).

Rab21 shows the highest homology with endocytic Rab5 and Rab22 (Stenmark and Olkkonen, 2001), and cells overexpressing wild-type or active variants of these proteins have enlarged early endosomes that label with antibodies against the early endosome associated antigen EEA1 (D’Arrigo et al., 1997; Kauppi et al., 2002). On the basis of these observations and our findings of colocalisation of Rab21 with the transferrin receptor, endocytosed EGF and indeed Rab5 and Rab22, we investigated whether a similar phenotype is observed in HeLa cells expressing Rab21. Both Rab21 wt and Rab21 Q78L colocalised with this Rab (Fig. 3D-F,G). The specificity of such localisation analysis was further highlighted by the observation that less than 10% of the Rab21 structures overlapped with Rab1a (Fig. 3G).

We therefore decided to screen a variety of other cell lines in an attempt to identify one expressing detectable levels of Rab21 protein. From these experiments, the hepatoma Hep3B cell line appeared to contain the highest levels of endogenous Rab21 protein as determined by immunofluorescence analysis. Double labellings with the anti-Rab21 and EEA1 antibodies revealed that both proteins colocalised on several punctate structures, in particular those in a juxtanuclear position (Fig. 5A-C, arrows). There were, however, several unique Rab21 and EEA1 structures, clearly showing that the colocalisation was only partial. Comparable to the HeLa cells, very little colocalisation of Rab21 with Lamp-1 was observed (not shown). We then repeated the GFP-Rab21 overexpression experiments in Hep3B cells to investigate whether the effect of Rab21 expression on EEA1 labelling was a feature exclusive to HeLa cells. Intensely fluorescent EEA1-positive structures were clearly seen in GFP-Rab21 wt expressing Hep3B cells, and these also labelled strongly with Rab21 (Fig. 5D-F, arrows). In addition, and similar to that seen in HeLa cells, these EEA1 structures were somewhat enlarged in the transfected cells, compared with nontransfected cells. Analysis of the localisation patterns of the GFP-Rab21 variants in these cells also revealed that they had similar phenotypes to those seen in HeLa cells (Fig. 5G-L), although the perinuclear labelling was less prominent. Altogether, these data argue for an early endosomal
localisation of endogenous Rab21 and suggest that endosome morphology is differentially responsive to overexpression of Rab21 and Rab21 mutants.

Detection of Rab21 subcellular distribution by immunogold labelling and electron microscopy

To more accurately determine the subcellular localisation of Rab21 and corroborate our fluorescent microscopy data, we prepared cryosections for subsequent immunolabelling of GFP-Rab21. Thawed cryosections from HeLa cells transfected, using calcium phosphate, with wild-type GFP-Rab21 were initially single labelled with polyclonal anti-GFP antibodies and secondary antibodies complexed to 15 nm gold. Electron microscopic observation of these sections showed that Rab21 labelled the membranes of Golgi cisternae and tubulo-vesicular elements close to these structures (Fig. 6). In particular, peripheral membranes of many endosomes were strongly labelled by Rab21 (Fig. 6B). We also observed by electron microscopy that Rab21 was often localised to cellular processes and the protein was seen on vesicular membranes and the plasma membrane of these structures (Fig. 6A). Plasma membrane labelling was, however, not confined to cell processes as clearly shown by the presence of the protein on the plasma membrane of the cell in Fig. 6D.

To confirm and extend our previous observations of colocalisation of Rab21 and endosomal markers, thawed cryosections were double labelled with antibodies against GFP and either EEA1, TfR or Hrs, a protein localised to endosomes and implicated in endosomal sorting (Clague and Urbe, 2001; Raiborg and Stenmark, 2002). The sequence of antibody labelling was changed for each combination in order to confirm true colocalisation. Rab21 and EEA1 colocalised predominantly on the outer membranes of early endosomal vesicles, vacuoles and tubules (Fig. 6C,D). Colocalisation of Rab21 with the TfR was also seen on endosomal structures (Fig. 6E-G) and, in addition, some labelling of tubulo-vesicular structures was observed in more perinuclear areas. It was also possible to observe the presence of Rab21 on membranes in close proximity to – and in some cases on – the Golgi cisternae, but these structures did not label for TfR or Hrs (Fig. 6E). Rab21 and Hrs were, however, localised on the same endosomal structures (Fig. 6H,I), although as shown in Fig. 6I, there were several examples of single-labelled structures. Together, these studies predominantly localised GFP-Rab21 to the early endocytic pathway and Golgi structures.

Transferrin uptake is inhibited in cells expressing GFP-Rab21T33N

Internalised transferrin traverses distinct Rab5, 4 and 11 domains in transit from the plasma membrane to recycling endosomes (Sonnichsen et al., 2000). Cells expressing mutants of these Rabs have numerous defects in transferrin traffic as it
attempts to cycle between the plasma membrane and the endocytic pathway. As Rab21 colocalises to differing degrees with these endocytic Rabs and in order to further pinpoint its endocytic location we investigated whether Rab21 colocalises with internalised transferrin, whether expression of Rab21 influences transferrin uptake and whether any colocalisation of Rab21 with transferrin is more pronounced at defined endocytic localities. Initially Hep3B cells were transfected with the GFP-Rab21 constructs and incubated with Texas Red-conjugated Tf (TxR-Tf) for 15 minutes at 37°C. After this time the cells were fixed and processed for fluorescence microscopy. We observed that many of the TxR-Tf structures colocalised with GFP-Rab21 wt in distinct vesicles (Fig. 7A-C) and that cells expressing Rab21 T33N had significantly lower levels of internalised transferrin compared with control cells (Fig. 7G-I). Expression of Rab21 wt or Q78L did not significantly affect transferrin uptake (Fig. 7A-F), and the transferrin receptor was normally distributed in cells expressing all three variants (not shown). To study the colocalisation of Rab21 wt and transferrin further, we repeated these experiments in HeLa cells and specifically contained the label in early endosomes by internalisation for 4 minutes at 37°C or for 1 hour at 16°C – a
condition that prevents exit of internalised material from early endosomes (Ren et al., 1998; van Dam et al., 2002). When early endosomes were specifically labelled with TXR-Tf after 4 minutes uptake, a significant number of distinct peripheral and perinuclear structures were seen to contain Rab21, EEA1 and the internalised ligand (Fig. 7J–M). Under these conditions we observed that 60% of the ligand was present in Rab21-positive structures, which is in good agreement with the degree of overlap after internalisation for 1 hour at 16°C (Fig. 7N).

We then investigated whether the transferrin that localises to early endosomes could be separated from Rab21 structures by chasing it to recycling endosomes. For this, TXR-Tf was internalised for 30 minutes at 37°C, followed by washing the cells and chasing the ligand for a further 30 minutes in the presence of an excess of unlabelled Tf. We observed that the degree of colocalisation dropped by 20%, indicating that some depletion of transferrin from Rab21 structures had occurred but that a significant fraction still remained associated with this protein (Fig. 7N).

**Endocytosis and degradation of epidermal growth factor is inhibited in cells expressing Rab21 T33N**

Cells overexpressing mutant and wild-type Rab5 as well as Rab22a have numerous endocytic defects, including an inability to efficiently traffic EGF from the plasma membrane to lysosomes (Kauppi et al., 2002; McCaffrey et al., 2001). Given the homology of Rab21 with Rab5 and Rab22, in combination with its localisation to early endosomes and its effects on endosome morphology and TXR-Tf uptake, we investigated the endosomal trafficking of EGF in cells overexpressing GFP-Rab21 variants. For this, Rh-EGF was internalised for various periods of time into transfected HeLa cells, which were then analysed by confocal fluorescence microscopy. Fig. 8 shows Rab21 localisation and Rh-EGF distribution in examples of nontransfected cells or those expressing GFP-Rab21 variants after 30 minutes of Rh-EGF internalisation. Cells expressing GFP-Rab21 wt and Rab21 Q78L had similar levels and distribution of internalised Rh-EGF compared with nontransfected cells (Fig. 8A–F). There was, however, a clear reduction in Rh-EGF fluorescence in cells expressing GFP-Rab21 T33N, and furthermore the ligand appeared to be restricted to relatively small, diffuse vesicles (Fig. 8G–I), similar to those seen with the internalised TXR-Tf. This prompted our quantification of the amount of internalised label in these cells and the numbers and fluorescence intensities of Rh-EGF-positive vesicles. This method of quantification was chosen over biochemical methods, as it allows measurements to be made from cells overexpressing only low-to-moderate amounts of the Rab21 proteins, and furthermore permits accurate comparison between transfected and nontransfected cells. Ten representative examples of nontransfected and Rab21 wt, Rab21 Q78L and Rab21 T33N expressing cells were selected for this purpose. Care was taken to avoid selecting cells expressing extremely high GFP-Rab21 levels and exposure times were standardised throughout; representative cells are shown in Fig. 8. The mean number of structures per unit area of 70×70 pixels corresponding to 10×10 μm were quantified, together with the mean fluorescence per structure and the total fluorescence per selected area. Table 1 confirms our visual observations in Fig. 8 and shows that expression of GFP-Rab21 T33N leads to a significant reduction in both the mean Rh-EGF fluorescence per structure and the total fluorescence in the cell, despite the presence of a greater number of Rh-EGF-positive structures. The reduction in mean fluorescence/structure in Rab21 T33N cells, compared with other cells, was approximately 30% for the two time points; however, the inhibition in total fluorescence increased from 30% after 15 minutes of internalisation to 60% after 30 minutes. This observed reduction in the intensities of the Rh-EGF structures in the Rab21 T33N expressing cells was, however, not a result of reduced binding of the ligand to cell surface receptors of these cells. When these experiments were repeated on ice, without warming the cells, comparable levels of bound EGF were observed (not shown). Live cell video microscopy (see supplementary material, Movies 4–6) of cells incubated with Rh-EGF at 37°C clearly show the difference in the levels of internalised ligand and the sizes of EGF containing structures between nontransfected cells and those expressing Rab21 wt, Rab21 Q78L and Rab21 T33N.

Two studies using different methods of analysis have shown that overexpression of wild-type, GTP binding and GTP hydrolysis mutants of Rab5 and Rab22a inhibit degradation of internalised EGF (Kauppi et al., 2002; McCaffrey et al., 2001). Either overexpression of all variants leads to defects in the ability of cells to deliver the ligand to lysosomal compartments.

### Table 1. Quantitative analysis of cellular Rh-EGF fluorescence

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Nontransfected</th>
<th>GFP-Rab21wt</th>
<th>GFP-Rab21Q78L</th>
<th>GFP-Rab21T33N</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structures/unit area</td>
<td>17.0±3.3</td>
<td>17.4±4.4</td>
<td>19.7±6.3</td>
<td>24.8±7.6**</td>
</tr>
<tr>
<td>Mean fluorescence/structure</td>
<td>140.3±17.1</td>
<td>139.8±19.8</td>
<td>127.1±18.9*</td>
<td>97.8±24.8****</td>
</tr>
<tr>
<td>Total fluorescence of all structures (×10³)</td>
<td>21.1±5.1</td>
<td>21.3±3.4</td>
<td>20.1±6.4</td>
<td>14.5±9.4*</td>
</tr>
<tr>
<td>30 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structures/unit area</td>
<td>20.0±5.8</td>
<td>19.0±3.1</td>
<td>20.2±4.4</td>
<td>24.6±8.2</td>
</tr>
<tr>
<td>Mean fluorescence/structure</td>
<td>137.6±20.3</td>
<td>136.3±13.2</td>
<td>143.5±18.1</td>
<td>99.8±12.4****</td>
</tr>
<tr>
<td>Total fluorescence of all structures (×10³)</td>
<td>18.0±8.4</td>
<td>19.2±9.6</td>
<td>15.6±6.8</td>
<td>6.5±2.4****</td>
</tr>
</tbody>
</table>

Quantitative analysis of Rh-EGF fluorescence in GFP-Rab21-expressing cells. HeLa cells transfected with GFP-Rab21 plasmids using calcium phosphate were incubated at 37°C for 15 and 30 minutes with 75 ng/ml Rh-EGF. The cells were washed on ice, fixed and analysed for the number of structures per unit area, the mean fluorescence per structure and the total fluorescence. Data shown are from ten cells, expressing moderate levels of either GFP-Rab21 wt, GFP-Rab21 Q78L, GFP-Rab21 T33N or nontransfected cells. At both time points, a similar number of Rh-EGF structures, with a similar fluorescence were observed in nontransfected cells and those expressing either GFP-Rab21 wt or GFP-Rab21 Q78L. However, cells expressing GFP-Rab21 T33N showed a distinct reduction in mean fluorescence per structure and in the total Rh-EGF fluorescence, despite containing more structures per unit area. Statistical significance is as follows: *P<0.05, **P<0.01 and ***P<0.001 compared with nontransfected cells.
Rab21 and early endosome dynamics

or their overexpression leads to secondary defects in lysosome function. We therefore utilised one of these assays to determine whether Rab21 expression also affected EGF degradation (Kauppi et al., 2002). Cells were allowed to internalise Rh-EGF for 1 hour at 37°C before chasing the label through the endocytic pathway using a further 2 hour incubation in its absence. As expected, owing to lysosomal degradation and/or recycling, almost all the fluorescence disappears in nontransfected cells after a 2 hour chase (not shown). Arrows in A-C point to Rab21 wt colocalisation with internalised TxR-Tf. (J-M) HeLa cells transfected with GFP-Rab21 wt plasmid were incubated with 50 nM TxR-Tf, for either 4 minutes at 37°C, 1 hour at 16°C or for 30 minutes at 37°C followed by washing and chasing for a further 30 minutes at 37°C in the absence of TxR-Tf, before fixing and analysis by fluorescence microscopy. Quantification of the number of TxR-Tf structures colocalising with Rab21 was determined for the different internalisation conditions. Error bars show mean and standard deviation between individual cells. Asterisk indicates statistical significance of \( P<0.001 \) compared with 4 minutes internalisation at 37°C.

Finally, we attempted to determine the location of this undegraded Rh-EGF in the Rab21 T33N expressing cells and whether it had been delivered to late endocytic structures. Following the internalisation regime described above, cells were fixed and immunolabelled for the late endosomal/lysosomal marker Lamp-1. As shown in Fig. 9L-M, we were unable to detect any colocalisation of the Rh-EGF with Lamp-1 in these cells, indicating that this Rab21 variant was interfering with the transport of the endocytosed ligand to the early endosome.
late endocytic compartments. We cannot exclude the possibility that the remaining EGF in these cells was the fraction that is normally recycled (Sorkin et al., 1989; Teslenko et al., 1987), but using a similar assay we did not observe any inhibition in the recycling of transferrin in Rab21 T33N expressing cells (not shown).

**Discussion**

In this study we describe the first functional characterisation of the small GTPase Rab21, and attribute a role to this protein in the dynamics of the early endocytic pathway. Rab21 was previously reported to be localised to the endoplasmic reticulum of nonpolarised epithelial CaCo-2 cells, and to be apically localised in polarised cells (Opdam et al., 2000). This preliminary study found partial colocalisation with the late endosomal/lysosomal marker α-glucosidase but no colocalisation with the transferrin receptor. By contrast, we find in HeLa and Hep3B cells, using immunofluorescence and immunoelectron microscopy, that Rab21 largely colocalises with, and affects the morphology of, early endosomes. In all cell lines, and typical for early endocytic Rabs, we also show that some Rab21 is localised at the plasma membrane. Our data show that internalised ligands are accessible to Rab21 vesicles after <6 minutes of internalisation and that EGF and Tf uptake are inhibited in cells expressing the GTP binding defective variant Rab21 T33N.

Immunofluorescence analysis revealed some Rab21 labelling in the perinuclear region, and we found a degree of overlap in this area with TGN markers. Furthermore, immunogold labelling and electron microscopy revealed that some of this perinuclear Rab21 was localised to Golgi stacks. Unlike Rab22, however, we did not observe any significant effects of Rab21 overexpression on Golgi morphology (Kauppi et al., 2002). Moreover, using a well-characterised assay of vesicular stomatitis virus glycoprotein G traffic (Kreis, 1986), we were unable to determine any effects of Rab21 wt or mutant expression on the delivery of this secretory protein to the plasma membrane (not shown). These combined observations suggest that Rab21 function is confined to the endocytic pathway, although we cannot currently exclude a possible requirement in an alternative pathway linking the Golgi complex and early endosomes.

Swollen EEA1-positive endosomes are also characteristic of cells expressing Rab5 and Rab22, and expression of their GTPase-defective mutants further enlarges these structures (D’Arrigo et al., 1997; Kauppi et al., 2002). Enlarged EEA1-containing structures were also a feature of cells expressing Rab21 wt but we were surprised to observe that these structures were not as prominent on cells expressing Rab21 Q78L. Interestingly, Rab21 Q78L predominantly labelled tubular structures, giving a reticular pattern of labelling that seemed to emanate from the perinuclear region. Although this may suggest that Rab21 is involved in tubule formation, these structures did not label with EEA1, Lamp-1 or internalised endocytic ligands, and we are currently unable to ascertain their identity using other cellular markers. Indeed, although this tubular network is reminiscent of the endoplasmic reticulum, there is little overlap of Rab21 Q78L with markers of this compartment (not shown). While this manuscript was in preparation, antibodies against Rab22a were shown to label tubular major histocompatibility complex class I (MHC)-positive structures whose prominence was enhanced in cells expressing the GTP hydrolysis mutant (Weigert et al., 2004). Contrary to previous studies suggesting a role for Rab22a in...
endocytosis from the plasma membrane to the lysosome (Kauppi et al., 2002; Mesa et al., 2001). Rab22a was shown to be required for recycling of MHC-I from early endosomes to the plasma membrane (Weigert et al., 2004). It was noted, although not shown in this study, that GFP-Rab21 did not localise to these organelles. Rat Rab22b was also localised to enlarged structures and tubular processes in HeLa cells expressing the YFP-variant and a Golgi-endosome trafficking function was postulated for this variant (Rodriguez-Gabin et al., 2001).

As expected, Rab21 T33N had minimal GTP-binding capacity and a significant fraction of the protein labelled the cytoplasm. Nevertheless, a surprising feature of Rab21 T33N-expressing cells was its strong prominence in a perinuclear area, and immunolabelling revealed significant overlap between this region and markers of the TGN. Expression of Rab21 T33N did not influence TGN morphology, and we are currently investigating the precise localisation of this variant together with its hydrolysis defective counterpart by electron microscopy.

Previous data have suggested that an endocyted molecule such as transferrin could traverse different Rab domains on the same endosome (Sonnichsen et al., 2000). Internalised transferrin was initially found to locate to mainly Rab5-positive structures after internalisation but then colocalised on the same endosome to a greater extent with Rab4 as it travelled on its way to Rab11 recycling endosomes en route to the plasma membrane (Sonnichsen et al., 2000). To further define the endocytic locality of Rab21 we compared the subcellular distribution of the YFP-tagged Rab21 wt protein with CFPTagged variants of several endocytic Rabs, and with Rab1a which is known to locate to and function at the endoplasmic reticulum and Golgi interface (Plutner et al., 1991). As expected, there was minimal overlap of Rab21 with Rab1a but extensive (>75%) overlap of Rab21 with Rabs 4a, 5a, 22a and also with Rab17, a Rab protein that is specific for epithelial cells and functions in apical recycling (Lutcke et al., 1993; Zacchi et al., 1998). Rab17 is phylogenically close to Rabs 5, 21 and 22 (Pereira-Leal and Seabra, 2001; Stenmark and Olkkonen, 2001) and was previously shown to be localised to cytoplasmic vesicles and tubules that were accessible to internalised probes in both polarised and nonpolarised cells (Peters and Hunziker, 2001; Zacchi et al., 1998). Consistent with our lack of colocalisation of Rab21 with markers for late endosomes and lysosomes, we did not see significant overlap with Rab7 (<20%), a mediator in late endosomal traffic (Feng et al., 1995; Gorvel et al., 1991).

Our studies show that in these overexpressing systems, Rab21 is confined to early as opposed to late endosomes, probably via a specific early endosomal binding partner that it may or may not share with the other early endocytic Rabs. The colocalisation of Rab21 with Rabs 9a and 11a was slightly higher than with Rab7 and these data are consistent with the previously described degree of colocalisation of Rab5 and Rab11 (Sonnichsen et al., 2000); Rab9 was not included in this study. Although Rabs 9 and 11 control different trafficking steps, the overlap with Rab21 may represent TGN compartments that are, to different extents, common to the three Rabs. As Rab21 colocalises predominantly with early endosomal Rabs it is likely that a single early endosome may contain at least four Rabs, most of which have at least two isoforms. Using confocal microscopy we could not confidently assign Rab21 to a specific endosomal domain that did not harbour other colocalised Rabs, and it is likely that electron microscopy and highly specific antibodies to the endogenous proteins will be required to prove that specific Rab domains exist.

Internalised EGF and transferrin labelled Rab21-positive compartments after <6 minutes internalisation, thus further confirming its localisation to early endosomes. Consistent with data in Rab5-expressing cells, we found that approximately 60% of transferrin structures in early endosomes, defined by short internalisation times or a 16°C block, also labelled with Rab21. After removal of labelled transferrin from the medium,
we could only partially chase the previously internalised ligand through the Rab21 structures, and after 30 minutes of chase, approximately 40% of the transferrin still colocalised with Rab21. We cannot currently prove that Rab21 expression does not affect transferrin delivery from early endosomes to Rab21-negative recycling endosomes but we did not observe any Rab21 effects on transferrin recycling. As the numbers are comparable, this 40% probably accounts for the fraction of Rab21 that colocalises with Rab11 and by default, transferrin. Transferrin and EGF endocytosis were stimulated and inhibited, respectively, in cells expressing Rab5 wild-type and Rab5 S34N (Barbieri et al., 2000; Bucci et al., 1992). Cells expressing Rab21 wt and Rab21 Q78L contained control levels of internalised EGF after 15 and 30 minutes uptake. However, using confocal fluorescence microscopy and subsequent quantification, we found that Rab21 T33N-expressing cells had a reduced capacity to internalise this ligand. In particular, we observed that in the Rab21 T33N expressing cells, despite there being a 45% increase in the actual amount of EGF-positive structures, there was a 30% decrease in the amount of internalised EGF both in the entire cell and in each endosomal structure compared to control cells. Analysis of both fixed and living cells containing internalised EGF clearly showed that the label was located in much smaller vesicular structures than those of nontransfected cells or those expressing active Rab21. Furthermore, we observed a similar ‘small endosome’ phenotype when we repeated these internalisation experiments with Tf in Hep3B cells. This suggests that Rab21 T33N-induced internalisation defects are common, albeit to varying degrees, to other cell lines and ligands. Small endosomes are also a feature of expressing Rab5 S34N (Stenmark et al., 1994), suggesting that these two proteins are inhibiting endosome dynamics via a common mechanism. We therefore suggest that Rab21, like Rab5, has a role in controlling endosome fusion and that expression of Rab21 T33N inhibits the fusogenic capacity of early endosomes.

Endocytic defects due to Rab21 expression were also observed downstream of early endosomes, manifested as an inhibition in the degradation of internalised EGF. Although the ligand was effectively degraded in cells expressing relatively low levels of Rab21 wt and Rab21 Q78L, in cells expressing higher levels a significant fraction remained undegraded. At the end of the chase period, cells expressing Rab21 T33N also had significant quantities of undegraded EGF in small vesicular structures diffusely located throughout the cytoplasm. Triple labelling experiments showed that these were not late endosomes or lysosomes, suggesting that Rab21 T33N inhibits the delivery of EGF to these late endocytic stations. Overexpression of wild-type Rab5 and Rab22 including their GTP-hydrolysis and GDP binding mutants (using viral expression systems) inhibit, to various extents, degradation of internalised EGF, while only expression of Rab4 and Rab7 GDP-binding mutants inhibit degradation (Kauppi et al., 2002; McCaffrey et al., 2001). In contrast to these data are separate findings (using calcium phosphate transfection) showing that Rab5 wt overexpression slightly increased EGFR degradation in response to EGF and that expression of Rab5 Q78L increased EGFR degradation twofold over nontransfected cells (Chen and Wang, 2001). Despite the fact that the experiments were performed in different cell lines, and using different expression vectors, it is surprising that EGF is inhibited from lysosomal degradation whilst degradation of its receptor is enhanced. Our observations of differing effects of Rab21 wt expression on EGF degradation suggest that the expression level is an important parameter in the interpretation of these data. More recent studies have suggested that the effects of Rab5 S34N expression on EGF endocytosis are downstream of the plasma membrane, as internalisation and degradation of EGF were unaffected and inhibited, respectively, in Rab5 S34N-expressing cells (Dinneen and Ceresa, 2004).

Interestingly, Rab5 S34N did not colocalise with the undegraded EGF, although the nature of this organelle was not characterised. Rab21 T33N labelling is rarely punctate and confined rather to the cytoplasm and Golgi; colocalisation analysis of Rab21T33N with other organelle markers, beyond the Golgi, is therefore extremely difficult. We find that the EGF-positive organelles in Rab21 T33N expressing cells are devoid of late endocytic/lysosomal markers; therefore, they may represent either a unique Rab21 T33N structure or a substation between early and late endosomes, such as endocytic carrier vesicles (Gruenberg et al., 1989). Studies are under way to further characterise these organelles.

In summary, Rab21 can now be added to the list of Rab members that localise to and function in the endocytic pathway. Although it shares several characteristics with Rab5 and Rab22, the distinct phenotypes of cells expressing Rab21 mutants suggests a unique role for this protein in endosome dynamics.

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