SARA, a FYVE domain protein, affects Rab5-mediated endocytosis

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Summary

Rab5, a member of the small GTPase family of proteins, is primarily localized on early endosomes and has been proposed to participate in the regulation of early endosome trafficking. It has been reported that phosphatidylinositol 3-kinases and FYVE domain proteins, such as EEA1, can be recruited onto early endosomes and act as Rab5 effectors. SARA (Smad anchor for receptor activation), also a FYVE domain protein, was initially isolated as a participant in signal transduction from the transforming growth factor β receptor. Overexpressed SARA has been found on EEA1-positive early endosomes. In this report, we show that endogenous SARA is present on early endosomes and overexpression of SARA causes endosomal enlargement. Functionally, SARA overexpression significantly delays the recycling of transferrin. The transferrin receptor distributed on the cell surfaces was also greatly reduced in cells overexpressing SARA. However, the internalization rate of transferrin is not affected by SARA overexpression. The morphological and functional alterations caused by SARA overexpression resemble those caused by overexpression of Rab5:GTP mutant Rab5Q79L. Finally, all SARA-mediated phenotypic changes can be counteracted by overexpression Rab5:GDP mutant Rab5S34N. These results collectively suggested that SARA plays an important functional role downstream of Rab5-regulated endosomal trafficking.

Key words: Rab5, FYVE domain, SARA, Transferrin, EEA1, Endosome

Introduction

The Rab family of small GTPase proteins plays central roles in intracellular membrane transport. These molecules are localized on distinct intracellular compartments and regulate the transport between specific organelles. The functional state of Rab proteins depends on their conformation, which is determined by their binding to either GDP (inactive state) or GTP (active state). It is believed that each transport step mediated by a Rab protein requires the active form of Rab to recruit the 'effector' protein to transduce signals for tethering/docking of vesicles to their target compartment, for vesicle budding, and/or for vesicle movement (for a review, see Zerial and McBride, 2001).

One of the best-characterized Rab proteins, Rab5, is mainly localized on early endosomes (Gorvel et al., 1991). Rab5 is known to be involved in both clathrin-coated vesicle-mediated transport from the plasma membrane to early endosomes and for homotypic early endosome fusion (Bucci et al., 1992; Gorvel et al., 1991). Overexpression of a GTPase-deficient Rab5 mutant Rab5Q79L resulted in enlarged endosomes, likely due to excess membrane fusion (Barbieri et al., 1996; Bucci et al., 1992; Stenmark et al., 1994). Furthermore, Rab5Q79L overexpression interferes with both receptor-mediated and fluid-phase endocytosis (Bucci et al., 1992; Li et al., 1995). So far, several Rab5 effectors have been isolated (Zerial and McBride, 2001). Among them, p150 and *p110β* are the regulatory subunit and catalytic subunit, respectively, of 2 different phosphatidylinositol (PI) 3-kinases (Christoforidis et al., 1999b). Interestingly, 2 other Rab5:GTP effectors – early endosome autoantigen 1(EEA1) (Simonsen et al., 1998) and Rabenosyn-5 (Nielsen et al., 2000) contain a double zinc-finger FYVE (Fab1p, YOTB, Vac1p, and EEA1) domain, which is known to bind to the phosphatidylinositol 3-phosphate (PtdIns(3)*P*), lipid product of PI3 kinase, with high specificity (Burd and Emr, 1998; Lawe et al., 2000; Stenmark and Aasland, 1999). It has thus been proposed that the Rab5:GTP as well as PtdIns(3)P generated on early endosomes provide a dual binding mechanism for the recruitment of EEA1 and Rabenosyn-5. In in vitro endosome fusion assays, both depletion of either EEA1 or Rabenosyn-5 inhibits this process (Lawe et al., 2000; Nielsen et al., 2000; Simonsen et al., 1998). In addition to their role in endosome fusion, EEA1 and Rabenosyn-5 exhibit their own unique structural and functional roles. For example, Rabenosyn-5, but not EEA1, associates with the Sec1 homologue hVPS45 and participates in endosome-lysosome trafficking (Nielsen et al., 2000).

SARA (Smad anchor for receptor activation) is also an FYVE domain protein. SARA was initially reported as a Smad2 interacting protein with roles in the recruitment of Smad2 and Smad3 to the ligand bound transforming growth factor beta (TGF-β) receptor. Thus, it plays a role in downstream signal transduction (Tsukazaki et al., 1998). Although overexpressed SARA has been shown to be located on endosome-like vesicular structures in cultured cells (Panopoulou et al., 2002; Seet and Hong, 2001; Tsukazaki et al., 1998), the exact localization of endogenous SARA and

whether SARA plays role in membrane trafficking have yet been fully investigated. In the present study, we demonstrate that endogenous SARA is localized to the early endosome, however, SARA labeling is not completely colocalized with EEA1 labeling. SARA overexpression induced endosomal enlargement and inhibited the recycling of transferrin (Tf) and reduced surface Tf receptor (TfR), phenotypes that have been described for Rab5:GTP overexpression (Stenmark et al., 1994). Both the morphological and functional changes caused by SARA could be corrected by co-expression of Rab5:GDP. These results collectively suggest that SARA is a novel player in the Rab5-regulated endosome trafficking pathway.

Materials and Methods

Reagents

Human Tf purchased from Sigma (St Louis, MO) was further purified by Sephacryl S-300 gel filtration. Iron-loaded diferric Tf and ^{125}I-Tf were prepared as described previously (Garippa et al., 1994; Yamashiro et al., 1984). Human Tf was labeled with the fluorescent dye Cy3 (Biological Detection Systems, Pittsburgh, PA) according to the manufacturer's instructions.

The following antibodies were used in this study: FLAG mAb (cloneM2; Sigma), EEA1 mAb (Transduction Laboratories, Lexington, KY), Rab5A rabbit antibody (S-19; Santa Cruz Biotechnology Inc, Santa Cruz, CA), and antibodies against the extracellular (B3/25; Chemicon) and the cytoplasmic (H68.4; Zymed) domains of the human TfR. Alexa-488 and Alexa-594 conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Cy5 conjugated anti-mouse IgG was purchased from Jackson ImmunoResearch Lab (West Grove, PA).

Cloning and constructs

In a previous study, we isolated positive clones from a two-hybrid screen of a bovine retinal cDNA library by using the C-terminal 39 residues of human rhodopsin as bait (Tai et al., 1999). One of these clones, when searched against the GenBank^{TM} database, encoded a partial sequence with a high degree of conservation to human SARA (Tsukazaki et al., 1998). This bovine SARA cDNA fragment was then used as a probe to screen a human retinal cDNA library in lambda gt10 (a kind gift of J. Nathans, Johns Hopkins University, School of Medicine, Baltimore, MD). Positive plaques were purified, and the cDNA inserts were cloned into pBluescript IIKS (Stratagene, La Jolla, CA) for sequencing. Sequences derived from a number of overlapping clones confirmed that they were human SARA cDNA fragments (Tsukazaki et al., 1998). Full-length SARA was subsequently generated by fusing 4 cDNA fragments into the eukaryotic expression vector pRK5. FLAG-tagged SARA and GFP-SARA were generated by placing full-length SARA 3′ to the FLAG peptide or EGFP, respectively. Detailed cloning procedures are available upon request. Expression vectors of Rab5, Rab5Q79L, and Rab5S34N were kind gifts from A. Francesconi (Albert Einstein Medical School, New York, NY).

Antibody production

A baculoviral expression vector encoding the human SARA fragment was generated by inserting the cDNA fragment encoding D112-V1324 of SARA into the pFastBacHTa vector (Invitrogen). Baculoviral protein encoding the His-tagged SARA peptide was produced in Sf9 cells and purified using a nickel column following the manufacturer's instructions.

Purified protein was then used as an immunogen for the production of rabbit antiserum (Cocalico, Reamstown, PA). To remove cross-reactivities, we passed the immunized serum through Sepharose columns conjugated with Sf9 cell lysates and His-tagged GST fusion proteins. The final flow-through was then affinity-purified on the His-tagged SARA conjugated Sepharose column, eluted with 0.1 M glycine, pH 2.8, and neutralized with Tris-Cl, pH 9.5. The resulting affinity-purified antibody is used for all experiments. Rabbit anti-SARA antibody recognized a ~140 kDa protein band from FLAG-SARA transfected human embryonic kidney (HEK) cell lysates. A protein band with the same molecular weight was also recognized by anti-FLAG antibody. SARA antibody did not recognize EEA1, which was identified by anti-EEA1 antibody as a ~180 kDa protein band on the same immunoblot.

Transfection and immunofluorescent staining of cell cultures

For transient transfection, HEK 293T cells and MDCK were transfected and immunolabeled as described previously (Chuang and Sung, 1998). Briefly, cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.04% saponin before antibody incubation.

PFA fixed, non-permeabilized cells were used to labeled surface TfR with anti-TfR antibody (B3/25), followed by Alexa594-conjugated anti-mouse antibody. Since there is no detergent was used, only the epitopes exposed on the cell surface had access to the antibody. In some experiments, after the primary antibody incubation, cells were washed, post-fixed, permeabilized, and incubated with anti-Rab5 antibody followed by the corresponding secondary antibodies for detection. The samples were examined on an Axioskop 2 epifluorescent microscope (Zeiss, Oberkochen, Germany) equipped with the appropriate filter sets, and images were captured by a SPOT 2 cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI). Alternatively, samples were examined by a confocal microscope (Zeiss LSM 510). Images were processed using Metamorph software and exported to Adobe Photoshop for printing.

The relative surface to total TfR ratio was determined as described in (Lampson et al., 2000). Briefly, cells grown on coverslips were incubated with Cy3-Tf (3 μg/ml) in serum-free medium (220 mM sodium bicarbonate, 20 mM HEPES pH 7.4) for 2 hours at 37°C to saturate the Tf/TfR pathway. The cells were subsequently transferred onto ice, washed three times with cold PBS-C/M and fixed with 4% PFA. The surface TfR was detected by B3/25 mAb followed by Cy5-conjugated anti-mouse antibody. Fluorescence microscopy was performed with a DMIRB inverted microscope (Leica, Deerfield, IL), with a cooled CCD camera (Princeton Instruments, Trenton, NJ). Images were acquired using a 63× 1.32 NA oil immersion objective. For quantification, total fluorescence of each fluorophore was summed over all cells in a field using Metamorph software (Universal Imaging, West Chester, PA), and nonspecific fluorescence (signals obtained from cells labeled only the Cy5-conjugated secondary antibody) was subtracted. Cy5/Cy3 ratios were measures of surface TfR normalized for the TfR expression level.

To generate stable HEK lines, 293S cells were transfected with the FLAG-SARA and neomycin expression vectors. Cells surviving in medium containing G418 (500 μg/ml) were selected by cloning rings, and positive clones were identified by immunofluorescent staining and immunoblot assays. The stable lines were maintained in DMEM/F12 medium supplemented with 5% calf serum and G418 (250 μg/ml). Based on the immunostaining results, more than 90% of the cells expressed FLAG-SARA in the early-passage cells. The percentage of FLAG-SARA expressing cells decreased during subsequent passages, presumably the toll of SARA overexpression. For experiments in this paper, we used cells before passage 5, at which time at least 70% of the cells are FLAG-SARA immunoreactive. Based on immunoblotting, we estimated that the level of SARA expression in the stable clone we used for this study is about 2-3-fold relative to the endogenous SARA.

Tf recycling assays

The Tf recycling rate was measured biochemically as described

previously (Johnson et al., 2001). Briefly, cells stably expressing FLAG-SARA or HEK cells were plated in gelatin-coated 24-well tissue culture plates for 2 days before the experiment. Cells were incubated with 3 μg/ml ^{125}I-Tf in serum-free medium for 2 hours at 37°C to achieve steady-state occupancy of the TfR. Cells were washed with medium 2 (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$) three times, followed by a 2 minute wash in mild acid wash solution (200 mM NaCl, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.0) and a 1 minute incubation with medium 2 to release surface-bound Tf. Cells were subsequently incubated in efflux medium (3 μg/ml unlabeled Tf and 100 μM iron chelator desferroxamine in serum-free medium) at 37°C for 0, 5, 10, 15, 20, or 30 minutes. At each time point, the efflux medium was collected and the cells were solubilized for gamma counting. The radioactivity in the efflux medium represents the ^{125}I-Tf released from the cells during the chase incubation, and the cell-associated radioactivity is the Tf remaining inside cells. Cells incubated with a 200-fold excess of unlabeled Tf were used to determine nonspecific binding of ^{125}I-Tf. The radioactivity of the efflux medium and solubilization solution was corrected by subtracting the average of the radioactivity in the background wells. To calculate the recycling rate constants, time points up to 30 minutes were used. The recycling rate constant is the slope of the natural logarithm plot of the percentage of Tf remaining associated with cells versus time.

Tf uptake assays

For the uptake experiments, HEK cells transfected with GFP-SARA (or together with Rab5S34N) were loaded with Cy3-Tf (3 μg/ml) in serum-free medium for 6, 15, or 120 minutes at 37°C. Cells were then placed on ice, washed with acidic buffer and then medium 2. Cells were subsequently fixed with 4% PFA for visualization. In the double transfection experiments, a 5:1 ratio of Rab5S34N- and GFP-SARA-encoding plasmids was used to ensure all GFP-SARA-positive cells were also Rab5S34N transfected. The expression of Rab5S34N in all GFP-SARA-positive cells was confirmed by the immunostaining of Rab5 on a duplicate coverslip.

To determine the Tf internalization rate constant, HEK control cells and HEK cells stably expressing FLAG-SARA were plated in gelatin-coated 6-well clusters. Cells were loaded with ^{125}I-Tf (3 μg/ml) in prewarmed serum-free medium for 2, 4, 6, 8, 10 minutes at 37°C and then transferred to ice for washing. Cells were rinsed twice in medium 2, followed by a 5 minute incubation in mild acid wash solution and a 5-minute incubation in medium 2. Cells were then solubilized in 1% Triton X-100 containing 0.1 N NaOH for gamma counting. In addition, counts derived from cells incubated with ^{125}I-Tf and excess unlabeled Tf (600 μg/ml) were considered as background and cells incubated with ^{125}I-Tf at 4°C for 2 hours were considered as total surface labeling. The internalized vs. surface ^{125}I-Tf were plotted over time to derive the internalization rate calculation (Wiley and Cunningham, 1982). Four duplicates were used for each time point in each experiment and three independent experiments were carried out.

Results

Endogenous SARA is found on early endosomes

Affinity-purified anti-SARA antibody was generated and used to detect the intracellular distribution of endogenous SARA. Immunofluorescent staining of HEK cells with anti-SARA antibody revealed punctate, endosome-like labeling throughout cells (Fig. 1A). Confocal analysis of HEK cells double labeled for SARA and early endosome marker EEA1 showed that these two molecules are both present on the majority of early endosomes. However, it is intriguing to note that the relative intensity of SARA and EEA1 on individual endosomes varied: some of the early endosomes display more SARA, whereas others display more EEA1 (Fig. 1A vs. 1B; Fig. 1D vs. 1E). In addition, SARA and EEA1 were not homogeneously distributed and not completely overlapping on individual endosomes (Fig. 1D-F).

SARA-mediated endosome enlargement requires active Rab5

In contrast to endogenous SARA, we found that the ectopically expressed SARA was distributed on much larger vesicular compartments, which were also Rab5- and EEA1-positive (data not shown). Consistent to the previous reports (Panopoulou et al., 2002; Seet and Hong, 2001; Itoh et al., 2002), this observation suggested that overexpressed SARA resulted in early endosome expansion.

The ability of SARA to induce endosome enlargement is similar to the phenotype described for the overexpression of a GTP hydrolysis-deficient mutant of Rab5 (Rab5Q79L) in cell

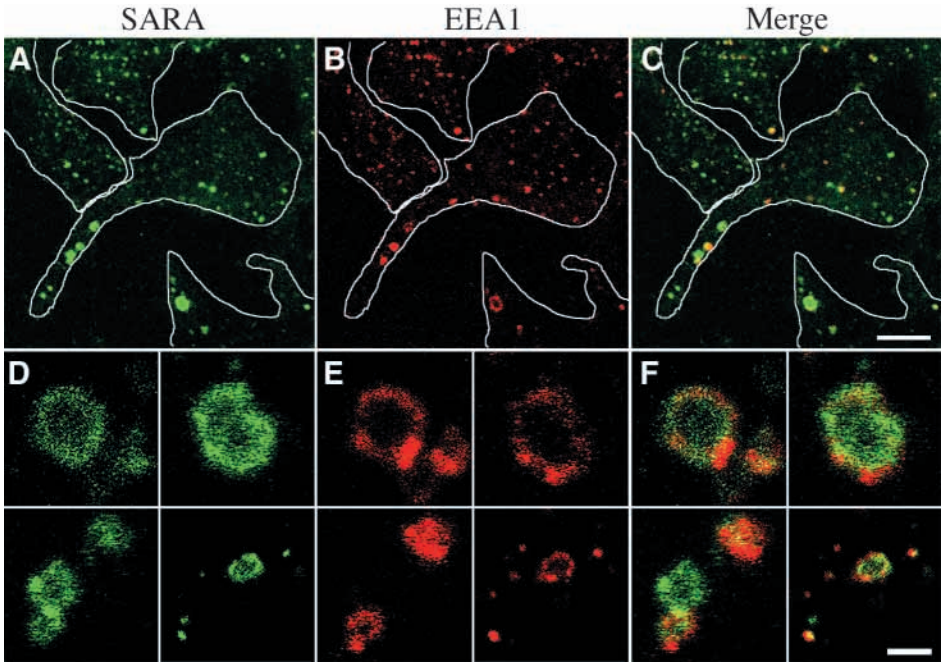

Fig. 1. Localization of endogenous SARA on early endosomal membranes. Low magnification (A-C, Bar, 10 μm) and 4 selective views of high magnification (D-F, Bar, 2 μm) images obtained from a single confocal section (0.6 μm) of HEK cells double labeled for SARA (A,D) and EEA1 (B,E). The images reveal heterogeneous pools of early endosomes displaying various levels of SARA and EEA1. Some early endosomes have a higher level of SARA, while others have a higher level of EEA1. SARA and EEA1 did not always overlap on the membranes of individual early endosomes.

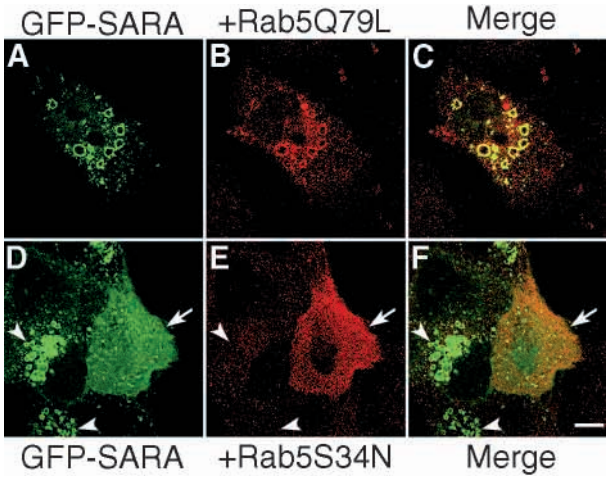

Fig. 2. Reversal of SARA-mediated endosome enlargement by Rab5S34N overexpression. MDCK cells cotransfected with GFP-SARA (green) with Rab5Q79L (A-C) or Rab5S34N (D-F) were fixed for Rab5 immunostaining (red). Rab5 transfected cells can be easily distinguished from the neighboring untransfected cells based on their higher level of Rab5 immunolabeling. Because the photographic exposure was chosen to highlight the transfected cells, the endogenous Rab5 labeling is negligible in these figures. (A-C) Rab5Q79L labeling is extensively colocalized with SARA on enlarged early endosomal membranes. (D-F) In contrast to the cells singly transfected with GFP-SARA, in which green fluorescence is observed on enlarged endosomal membranes (arrowheads), GFP signals are distributed diffusely in the cytosol and on small puncta in the cells co-transfected with GFA-SARA and Rab5S34N (arrow). Bar, 10 μm.

cultures (Stenmark et al., 1994; Barbieri et al., 1996). To determine the functional relationship between SARA and Rab5, MDCK cells were cotransfected with GFP-SARA together with Rab5Q79L (Rab5:GTP) or Rab5S34N (Rab5:GDP) and fixed for immunolabeling. In these experiments, Rab5Q79L was extensively colocalized with SARA on the membranes of enlarged endosomes (Fig. 2A-C). The sizes of these enlarged endosomes (2-9 μm) were comparable to those induced by overexpression of Rab5Q79L alone (data not shown). Although there was a great heterogeneity among the vesicle sizes in these transfected cells, we consistently found that the large vesicles induced by Rab5Q79L and GFP-SARA overexpression were significantly larger than those induced by overexpressing GFP-SARA alone [1-5 μm, compare Fig. 2A vs. 2D (arrowheads)]. In striking contrast, the large endosomes caused by GFP-SARA overexpression disappeared in the Rab5S34N cotransfected cells (Fig. 2D, arrow). Instead, in the double transfected cells, GFP-SARA was mainly detected in the cytosol and on small membrane vesicles. These results indicated that Rab5:GTP is a prerequisite for SARA-mediate endosomal membrane enlargement.

However, unlike the two FYVE domain proteins EEA1 and Rabenosyn-5, which interact with Rab5:GTP directly (Simonsen et al., 1998; Nielsen et al., 2000), no specific interaction between SARA and Rab5Q79L could be detected despite our repeated attempts with both two-hybrid and co-immunoprecipitation assays (data not shown). These results together suggested that the effect of Rab5 mutants on SARA distribution was most likely through indirect interaction, such as via the PtdIns(3)*P*, rather than the direct interaction between Rab5 and SARA.

Tf trafficking was affected by SARA overexpression

To access the functional consequences of SARA overexpression, we examined the endocytosis and recycling of Tf in SARA overexpressing cells. To examine Tf uptake, GFP-SARA transfected HEK cells were incubated with Cy3-Tf at 37°C for various time points before the cells were fixed for visualization. Within 6 minutes, Cy3-Tf was found in the early endosomes peripheral to the plasma membrane throughout the untransfected cells (Fig. 3A). In contrast, very little or no Cy3-Tf was detected in the SARA overexpressing cells at this time point. However, Cy3-Tf could be detected in the SARA-positive enlarged endosomes 15 minutes after Tf uptake. After 2 hours of loading, Tf reached a steady-state occupancy of the endocytic pathway and was distributed throughout the early endosomes and the recycling endosomes in the untransfected control cells. On the other hand, Tf was largely concentrated in the SARA-positive endosomal compartments in the GFP-SARA transfected cells. Double labeling of TfR showed that the internalized TfR was also primarily associated with Tf in the SARA-positive compartments (data not shown), suggesting that Tf and TfR remained associated in the expanded early endosomes.

To determine if the internalization rate of Tf was affected by SARA overexpression, we compared the internalization rate constant of HEK cells stably expressing FLAG-SARA and untransfected HEK cells. In these assays, cells were loaded with ^{125}I-Tf for 2-10 minutes at 37°C before harvesting. The ratio of radioactivity derived from the internalized Tf to the surface Tf was plotted as a function of time to determine the K_i, the endocytic rate constant. One example is shown in Fig. 3C: a SARA stable line and the parental HEK cells internalized Tf at approximately the same rate. The values for K_i were 0.12±0.02 minute^{-1} and 0.13±0.02 minute^{-1} for SARA stable line and HEK cells, respectively (4 duplicates for each experiments and 3 independent experiments).

We next examined the amount of surface TfR by immunolabeling with B3/25 mAb, which recognizing the extracellular domain of TfR. As shown in Fig. 4A,B, the punctuate surface labeling of TfR was significantly reduced in the GFP-SARA overexpressing cells compared to the untransfected cells. Quantitative measurement (see Experimental Procedures) showed that whereas the total TfR receptor levels (i.e. 2 hour Tf uptake at 37°C) were almost identical between the SARA-overexpressing cells and control cells, GFP-SARA overexpressing cells only displayed about 70% of surface TfR relative to that of control cells (Fig. 3D). The reduced surface expression of the TfR in SARA expressing cells likely accounts for the failure to detect Tf uptake into SARA expressing cells during a 6 minute pulse with fluorescent Tf (Fig. 3A).

The reduction in surface TfR is not due to increased degradation of the TfR since the half-life of TfR is about the same in control cells and SARA-expressing stable cells (data not shown). Thus, the decrease in cell surface receptor does not reflect receptor degradation.

The above results collectively indicated that over-expression

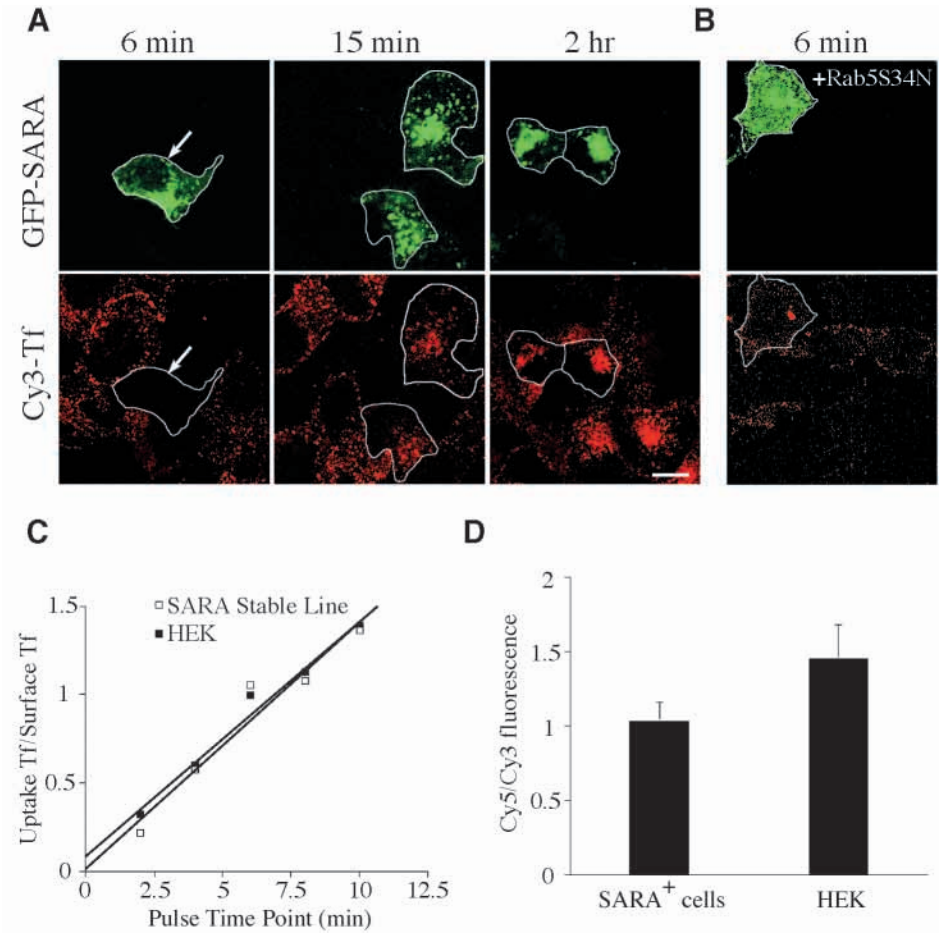

Fig. 3. Effect of SARA on Tf endocytosis. (A) GFP-SARA (green)-transfected HEK cells were loaded with Cy3-Tf (red) at 37°C for 6, 15 or 120 minutes before the cells were chilled, washed and fixed for GFP and Cy3 visualization. At 6 minutes, Cy3-Tf was readily detectable on small endosomes in the untransfected control cells, but not in the GFP-SARA transfected cells (arrows). At 15 minutes, Cy3-Tf becomes detectable in the GFP-SARA transfected cells. After the 2-hour loading, Cy3-Tf reached a steady-state occupancy and was distributed on both early and recycling endosomes in the control cells, whereas Cy3-Tf was largely concentrated in the SARA-positive compartments of the transfected cells. Bar, 10 μm. (B) HEK cells cotransfected with GFP-SARA and Rab5S34N were loaded with Cy3-Tf for 6 minutes before fixation. The level of internalized Cy3-Tf is indistinguishable between the double-transfected cells (indicated by cytosolic GFP-SARA signal) and the neighboring untransfected cells. (C) SARA stable transfectants and HEK cells grown in 6-well dishes were incubated with ^{125}I-Tf at 37°C for the time indicated. The amounts of total, internalized and surface-associated radioactivity were determined. Data are plotted as described in Wiley and Cunningham (Wiley and Cunningham, 1982), such that the slope is equal to the endocytic rate constant, K_i. Results shown are from a single experiment and are representative of those obtained on three separate occasions. (D) SARA-transfected cells and HEK cells grown on coverslips were loaded for 2 hours with Cy3-Tf in serum-free medium. The cells were then fixed and the surface TfR was labeled with Cy5 by indirect immunofluorescence. Quantitative data was obtained by summing the Cy3 and Cy5 fluorescence in each field (>20 cells per field), taking the ratio and averaging over multiple fields. The ratio of Cy5/Cy3 fluorescence, the index of the surface/total TfR, was obtained from the average of three independent experiments (means±s.e.m.).

of SARA slows the return of TfR to the cell surface. To test this possibility, GFP-SARA transfected HEK cells were incubated with Cy3-Tf for 2 hours at 37°C to reach a steady-state, and then chased in the presence of excess unlabeled Tf and iron chelator desferroxamine for various time periods. Cells fixed immediately after the 2 hour-Cy3-Tf loading showed that the majority of Tf had accumulated in the SARA-containing enlarged endosomes. In control cells about half of the internalized Cy3-Tf was recycled into the medium during a 10 minute chase incubation (Fig. 5A). Cy3-Tf recycling was significantly inhibited by SARA overexpression at all chase time-points examined. After a 30 minute chase, there was essentially no Cy3-Tf detectable in the untransfected cells, whereas an appreciable amount of Cy3-Tf remained in the SARA-positive endosomal compartments (Fig. 5A). These results are consistent with the model that Tf recycling was inhibited by the overexpression of SARA.

To quantify the Tf recycling rate of SARA expressing cells biochemically, we assayed the Tf efflux in SARA-expressing stable lines and HEK cells. In these experiments, cells were first incubated with ^{125}I-Tf for 2 hours at 37°C. After a mild acid wash to remove surface-bound ligand, cells were incubated in medium containing desferoxamine and unlabeled Tf for various time periods. Release of ^{125}I-Tf into the medium was calculated as a percentage of total intracellular Tf at 0 minute. As shown in a representative experiment (Fig. 5C), the SARA stable line released Tf at a significantly lower rate. In data pooled from 3 experiments, we observed the recycling constant of ^{125}I-labeled Tf was approximately 32% lower in the SARA-expressing cells compared to the control cells (Fig. 5D). The degree of Tf recycling rate reduction was consistent with the degree of reduction of surface TfR levels (~30%; Fig. 3D) in SARA overexpressed cells.

Rab5S34N overexpression suppresses SARA-mediated effects of Tf trafficking

The phenotypes caused by SARA overexpression (i.e. reduced surface TfR, and slower Tf recycling) were similar to those described for Rab5Q79L overexpression (Stenmark et al., 1994). To test whether SARA is involved in the Rab5-mediated Tf/TfR trafficking, we asked whether co-expression of the dominant-negative Rab5 mutant (Rab5S34N) can attenuate the phenotypes resulting from SARA overexpression.

In the Tf uptake experiments, the amount of Cy3-Tf internalized into cells expressing both GFP-SARA and Rab5S34N (indicated by the cytosolic distribution of GFP-SARA) in a 6 minute pulse was undistinguishable from that in neighboring untransfected cells (Fig. 3B). This is in contrast to SARA singly transfected cells, in which little internal Tf is detected in a 6 minute pulse (Fig. 3A).

In the surface TfR labeling experiments, cells overexpressing Rab5Q79L, but not Rab5S34N, displayed significantly lower levels of surface labeling of TfR compared to the neighboring cells (Fig. 4C,D and Fig. 4E,F). This is consistent with the biochemical experiment results of previous report (Stenmark et al., 1994). In contrast to the GFP-SARA singly transfected cells, the level of surface TfR labeling for Rab5S34N/GFP-SARA-overexpressing cells was very similar to that of untransfected control cells (compare Fig. 4A,B with Fig. 4G,H). Finally, co-expression of Rab5S34N with GFP-SARA redirected Cy3-Tf recycling back to the surface with kinetics similar to those of control cells. For example, at the 30-minute chase time point, almost all Cy3-Tf had exited from Rab5S34N/GFP-SARA double transfected cells (Fig. 5B).

Discussion

SARA- and Rab5-mediated early endosome enlargement

The FYVE domain is a conserved sequence present in more than 30 proteins in species from yeast to mammals. The major functional role of the FYVE domain proteins characterized thus far is membrane trafficking. In the present paper we showed that SARA is not an exception. However, SARA exhibits several distinct phenotypes related to its roles in the endocytic membrane trafficking. First, overexpression of SARA is sufficient to form enlarged endosomes. In addition to SARA, Endofin, a closely-related homologue of SARA, shares the ability to enlarge endosomes when it is overexpressed in tissue culture (Seet and Hong, 2001). In contrast, although EEA1 and Rabenosyn-5 are involved in endosome fusion in vitro, overexpression of these two molecules alone did not result in enlarged endosomes in culture (Patki et al., 1998). Based on the tethering roles proposed for EEA1 and Rabenosyn-5 (Christoforidis et al., 1999a; Nielsen et al., 2000), it is possible that the oligomerization of SARA could contribute to the early endosomal membrane fusion and/or expansion.

The enlarged endosome phenotype of SARA resembles the phenotype described for cells overexpressing Rab5Q79L. Furthermore, SARA-mediated endosomal swelling can be reversed by the overexpression of Rab5S34N. These results strongly argue that Rab5:GTP is required for recruitment of SARA onto early endosomal membranes and, in turn, for the morphological and functional alteration of the early endosome. It is intriguing that the extent of endosome enlargement appears to be greater in the Rab5Q79L expressing cells than in the SARA expressing cells. The higher degree of endosome fusion caused by Rab5Q79L overexpression can be explained by the existence of multiple Rab5 effectors, which might cooperatively enhance the endosome fusion events. However, at the present time, there is no evidence to rule out the possibility that an alternative mechanism is involved for SARA-mediated endosome swelling. For example, while Rab5Q79L overexpression is proposed to enhance homotypic fusion between early endosomal membranes, excess SARA may disassemble the coated protein on early endosomes and thus inhibit budding.

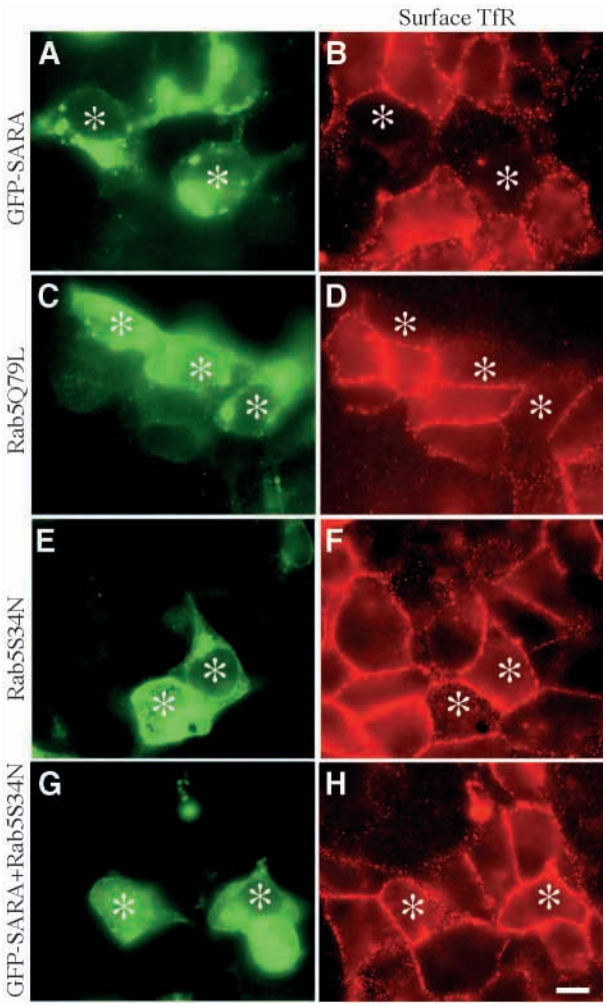

Fig. 4. The effect of overexpression of SARA and Rab5 mutants on surface TfR distribution. HEK cells transfected with GFP-SARA (A,B), Rab5Q79L (C,D), Rab5S34N (E,F) and both GFP-SARA and Rab5S34N (G,H) were fixed. Without permeabilization, the cells were labeled for surface TfR using an antibody recognizing the extracellular domain of TfR (B3/25), followed by Alexa594 anti-mouse IgG (B,D,F,H). Cells overexpressing GFP-SARA and Rab5Q79L, but not Rab5S34N, appear to have reduced surface TfR labeling (*). In contrast, cells double-transfected with GFP-SARA and Rab5S34N have similar levels of surface TfR compared with the neighboring cells (*). Bar, 10 μm.

Alteration in receptor-mediated endocytic trafficking by SARA overexpression

In addition to morphologic changes in early endosomes, overexpression of Rab5Q79L also causes a decrease in Tf recycling and the number of surface TfRs (Stenmark et al., 1994). However, the effector of these Rab5 functions has yet been identified. In the present report, we showed that SARA overexpression affects Tf and TfR trafficking in a similar fashion as Rab5Q79L overexpression. Furthermore, all these phenotypes

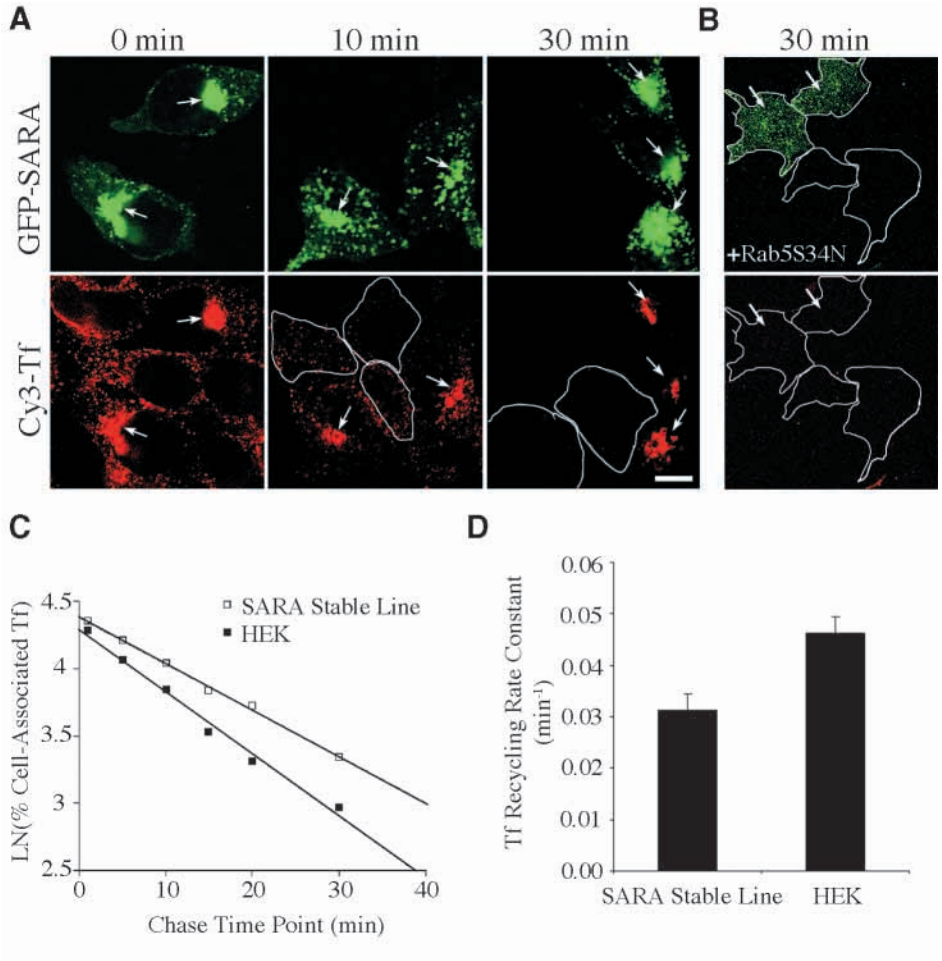

Fig. 5. Reduced Tf recycling in cells overexpressing SARA. (A) HEK cells transiently transfected with GFP-SARA were loaded with Cy3-Tf at 37°C for 2 hours and chased in the presence of excess unlabeled Tf and desferroxamine for 0, 10, and 30 minutes before the cells were chilled, fixed and observed. At all time points, Cy3-Tf was mainly accumulated in the GFP-SARA positive endosomal compartments (arrows). (B) HEK cells double-transfected with GFP-SARA and Rab5S34N were preloaded with Cy3-Tf for 2 hours and chased for 30 minutes. The double transfected cells (indicated by cytosolic GFP signal) release Cy3-Tf in a manner indistinguishable from the neighboring cells. Bar, 10 μm. (C) The ^{125}I-labeled Tf was loaded on HEK stably expressed SARA or HEK cells for 2 hours before the chase. The natural logarithm of the percent of Tf remaining inside the cell versus the chase time from one experiment is shown. (D) The mean±s.e.m. of the recycling rate constants from three independent experiments are presented. The Tf recycling rate constant of the SARA stable line is significantly slower than that of HEK control cells (P<0.01, two-tailed Student's t test).

caused by SARA can be reversed by co-expression of Rab5S34N. Taken together, these results argue for a functional link between SARA and the Rab5-mediated endosomal trafficking pathway. Namely, the Rab5Q79L-mediated Tf/TfR recycling delay is likely to be mediated through SARA. Nevertheless, we do not refer to SARA as a Rab5 effector because all other Rab5 effectors reported so far, but not SARA, can directly interact with Rab5 in in vitro binding assays.

It has been shown that Rab5Q79L overexpression increases the Tf internalization rate, whereas SARA overexpression has little or no effect on the Tf internalization rate. This is consistent with the involvement of Rab5 in the transport of clathrin-coated vesicles from the plasma membrane, whereas SARA is not detectable on the plasma membrane.

The early endosome is a highly dynamic structure. Constant fusion and fission are required to maintain the homeostasis of early endosomal compartments. Abnormal accumulation of Tf and TfR in the SARA-induced large early endosomes, but not the recycling endosomes, suggests that perturbation of early endosome morphology may affect the temporal and/or spatial cues for the transit from early endosome to recycling endosome. This interference may explain the slowdown of Tf and TfR recycling back to the cell surface.

Endosomal localization of SARA

Several lines of evidence suggest that the endosomal membrane distribution of SARA relies largely on the interaction between the FYVE domain and PtdIns(3)*P*. First, SARA displayed a cytosolic distribution in response to wortmannin (Panopoulou et al., 2002). Second, deletion of FYVE domain (Tsukazaki et al., 1998; Panopoulou et al., 2002; Itoh et al., 2002) results in a complete removal of its membrane localization. Finally, it has been reported that the first conserved cysteine at position 603 is critical for the FYVE domain structure (Gaullier et al., 2000; Seet and Hong, 2001). GFP-SARA mutant GFP-SARA^{C603S}, in which the cysteine-603 residue in the FYVE domain was mutated to serine, showed a completely cytosolic distribution (Y.H. and C.H.S., unpublished).

Recent data suggested that the FYVE domain of SARA (aa574-aa660) itself binds to PtdIns(3)*P* with a high affinity (K_d=30 nM) (Panopoulou et al., 2002). This affinity is higher than that detected between PtdIns(3)*P* and the FYVE domain of EEA1 (50 nM) (Gaullier et al., 2000). This affinity difference may be sufficient to explain why the FYVE domain of EEA1 (aa1336-aa1411) is not sufficient for its endosomal localization (Lawe et al., 2000). Instead, the dual interaction of EEA1 with PtdIns(3)*P* as well as Rab5 may be needed to increase specificity and stability for the association between EEA1 and endosomal membranes (Christoforidis et al., 1999a; Simonsen et al., 1998; Lawe et al., 2000).

In contrast to the Rab5 effectors isolated so far, SARA did not display a detectable interaction to Rab5:GTP in yeast two-

hybrid and co-immunoprecipitation assays (data not shown). Whether these negative results are resulted from weak or transient interaction between these two molecules or through a common binding partner is presently unclear. However, because our data showed that overexpression of Rab5:GDP suppresses the endosomal membrane localization of SARA as well as all the SARA-mediated functional phenotypes, we thus propose that Rab5:GTP stimulates the local enrichment of PtdIns(3)*P* on early endosomal membranes, and SARA is subsequently recruited to the early endosome based a single-mode interaction through PtdIns(3)*P*.

Within the past few years, increasing numbers of FYVE domain proteins have been localized to early endosomes. Why are multiple FYVE domain proteins needed by a single early endosome? And how do these FYVE domain proteins participate in Rab5-mediated endosomal functions in a coordinated manner? In the case of EEA1 and Rabenosyn-5, although both of them share roles in endosome fusion, EEA1 is unable to rescue the endosome fusion suppression caused by the depletion of Rabenosyn-5 (Nielsen et al., 2000). This suggests that EEA1 and Rabenosyn-5 serve somewhat distinct roles in these processes. In addition, Rabenosyn-5 plays a role in lysosomal trafficking of cathepsin D, which EEA1 does not (Nielsen et al., 2000). One model that has been proposed is that there are functional subcompartments on early endosomes, and each subcompartment participates in a different aspect of endocytic trafficking (De Renzis et al., 2002). We have observed that endogenous SARA and EEA1 are indeed distributed on distinct microdomains of early endosomes. In addition, the stoichiometry of these two molecules, indicated by the labeling intensity, also appears to vary among different pools of early endosomes. These observations suggest the possibility that different FYVE domain proteins may function differently based on their spatially distinct distributions and the level of protein expression.

SARA, a functional link between signal transduction and vesicular trafficking

SARA has been suggested to be involved in the TGF-β receptor mediated signal transduction pathway. SARA recruits Smad2 and Smad3 to the TGF-β receptor upon receptor stimulation. The Smads are then phosphorylated by the activated receptor kinases. The phosphorylation of Smad2 and Smad3 enable them to bind Smad4. The resulting heteromeric complex is subsequently translocated to the nucleus, where it controls the transcription of target genes (Tsukazaki et al., 1998). Although ectopicically expressed SARA has been found on endosomes in previous reports (Seet and Hong, 2001; Panopoulou et al., 2002), the present report, for the first time, confirms that the endogenous SARA is indeed localized on early endosomes. Together with others (Panopoulou et al., 2002; Itoh et al., 2002), these results support the model that the TGF-β receptor signaling is taken place on the endosomal membranes.

In addition to its signal transduction role, this report suggests that SARA also plays an important role in the dynamic morphology and function of endosomes, arguing that SARA is a molecule linking between membrane trafficking and signal transduction on endosomes. Similar to SARA, the FYVE domain protein-hepatic growth factor-regulated tyrosine kinase substrate (Hrs) has also been linking between the TGF-β/Smad signalling pathway and endosomal membrane trafficking (Raiborg et al., 2001b). Nevertheless, the endosomal targeting of Hrs is *via* a Rab5-independepent pathway (Raiborg et al., 2001a).

In addition to its role in downregulating surface receptors, endocytosis has been recently recognized as a mechanism tightly associated with the signaling pathway. One prominent example is that the internalization of G-protein coupled receptor (GPCR) is targeted to endosome, via the β-arrestin- and the clarthin-dependent pathway, on which β-arrestin recruites and activates certain members of mitogen-acitvated protein kinase (MAPK) cascades (Pierce and Lefkowitz, 2001). Furthermore, it has been shown that Rab5 plays roles in the internalization, endosomal sorting and recycling of a number of GPCRs (Iwata et al., 1999; Seachrist et al., 2000; Seachrist et al., 2002). It would be of great interest to investigate whether SARA also participates roles in the signal transduction pathways of GPCRs on endosomal compartments.

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