Endofin, an Endosomal FYVE Domain Protein*

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KIAA0305 is an uncharacterized member of the FYVE domain protein family. It is closely related to SARA, with about 50% identity in the carboxyl-terminal 800-amino acid region. Indirect immunofluorescence microscopy using polyclonal antibodies raised against KIAA0305 revealed that it is enriched in early endosomes. The Myc-tagged version is also faithfully targeted to the early endosome. We have tentatively called KIAA0305 endofin (for endosome-associated FYVE-domain protein). The association of endofin with endosomes is mediated by its FYVE domain because deletion mutants lacking the central FYVE finger motif are distributed in the cytoplasm. In addition, a single point mutation in the FYVE finger motif at cysteine residue 753 (C753S) is sufficient to abolish its endosomal association. Its endosomal localization is also sensitive to the phosphatidylinositol 3-kinase inhibitor, wortmannin. Using in vitro liposome binding assays, we demonstrate that Myc-tagged endofin associates preferentially with phosphatidylinositol 3-phosphate, whereas the C753S point mutant was unable to do so. We also show that endofin co-localizes with SARA but that they are not associated in a common complex because they failed to co-immunoprecipitate in co-expressing cells. Endofin also does not associate with Smad2 nor behave like SARA in affecting transforming growth factor-β signaling. At high levels of expression, both endofin and SARA can cause an endosome aggregation/fusion effect. In COS7 cells, which can support high levels of exogenous protein expression, both proteins can also cause other structural anomalies in the endocytic pathway, as represented by enlarged vesicular structures. These endosomal aggregates/fusions accumulated endocytosed epidermal growth factor. Taken together, this report provides evidence to suggest that endofin and the highly related SARA are endosomal proteins with potential roles in regulating membrane traffic.

The movement of proteins between different membrane compartments of a cell requires the process of intracellular membrane trafficking. This is a multistep process that culminates in the transport of cargo to its intended destination via membrane-bound intermediates such as vesicles. Specificity of the system is implicitly critical, and protein-lipid interactions may contribute to the regulation of critical steps in the pathway. The role of protein-lipid interactions in regulating endocytic events has recently gained much support. An intensely researched area is the role of phosphoinositides or phosphorylated species of phosphatidylinositol (PI)1 in membrane trafficking (for reviews see Refs. 1 and 2). One of the most interesting phosphoinositides is phosphatidylinositol 3-phosphate (PI3P). PI3P is a product of phosphatidylinositol 3-kinase (PI 3-kinase) activity. One of the direct protein targets of PI3P has recently been identified to be the early endosomal autoantigen 1 (EEA1). This protein binds to PI3P via a conserved motif called the FYVE domain. This domain is so named based on the four proteins first identified to contain it: Fab1p, YOTB, Vac1p, and EEA1 (3). The FYVE domain is a highly conserved zinc-binding domain characterized by the presence of eight conserved cysteine residues, the third of which is flanked by characteristic basic amino acids: CX4CX3−9RRHHCRXCCXα6CX4−48CX2C (where X represents nonconserved amino acid residues). A comparison of the structures of various FYVE domain-containing proteins suggests that the FYVE domain is a modular domain that can function independently of its location in the protein.

The involvement of the FYVE domain in membrane trafficking is evidenced by the characterization of several mammalian FYVE domain-containing proteins such as EEA1, Hrs-2, Rabip4, and Rabenosyn-5 (for a review see Ref. 4). The prototype EEA1 has been shown to interact with Rab5 and to mediate fusion and docking of early endosomes (5–7). Mammalian Hrs-2 is highly expressed in neuronal cells and is implicated in regulating exocytotic vesicular transport at the synapse by binding SNAP-25 (8). Rabip4 is reported to control early endosomal traffic between recycling and sorting endosomes (9), whereas Rabenosyn-5 is involved in the regulation of early endosome fusion (10). In addition, several yeast FYVE domain proteins such as Vac1p, Vps27p, and Fab1p have been shown to play vital roles in membrane trafficking events ranging from the regulation of endosome docking or fusion, endosome maturation, and vacuolar membrane efflux or degradation (for reviews see Refs. 2 and 11). FYVE domain proteins have also been implicated in signal transduction. Hrs-1 was initially identified as a protein that is activated in response to hepatocyte growth factor and binds to signaling molecules such as STAM and Smads (12–14). SARA (for Smad anchor for receptor activation) was first identified as a mediator of the TGF-β signal transduction pathway via its ability to associate with Smads 2 and 3 (15).

The FYVE domain is thought to regulate endosomal or vacuolar trafficking and/or signal transduction by targeting proteins containing them to membranes enriched in PI3P, thereby

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delivering them to the correct intracellular location where they can exert their action. This view is supported by the observation that the majority of the characterized mammalian FYVE domain proteins including Ankhzn (16), DFCP1 (17), EEA1 (3), Rabenosyn-5 (9), Rabip4 (10), and SARA (15) are localized in early endosomes or vesicular structures in the cell. Of these proteins, the FYVE domain of Rabenosyn-5 is clearly demonstrated to be sufficient to target the protein to endosomal membranes (10). Furthermore, mutation or deletion of the FYVE domain in EEA1 or SARA has been shown to cause mis localization of the protein as well as abrogate its ability to regulate endocytosis or signal transduction, respectively (5, 15). In addition, a recent report utilizing a double FYVE domain derived from Hrs-1 fused to an enhanced green fluorescent protein reporter revealed the specific recruitment of this fusion protein to early endosomes. Together, these data support the notion that the FYVE domain functions by recognizing target membranes presumably enriched in PI3P (18). However, in apparent contradiction to this view, there are instances where the FYVE domain does not appear to be involved in targeting proteins to the early endosomes. One example is Frabin (EGD1-related F-actin-binding protein), which is not located in endosomes but co-localizes with F-actin instead (19). Fgd1, in turn, has been localized in the cytoplasm, the subcortical actin cytoskeleton, and the Golgi apparatus, and its intracellular distribution does not appear to depend on the FYVE domain (20). Also, PIKfyve is reported to co-localize primarily with Golgi-to-late-endosome markers (21). In the case of Hrs-1, its endosomal localization is not mediated by the FYVE domain but by a 100-amino acid sequence in the carboxyl-terminal region of the molecule (22). These findings suggest that the intracellular distributions of FYVE domain proteins can be highly variable and highlight the importance of determining the localization of new FYVE domain proteins and the underlying mechanism as an important step in understanding their biological roles in the cell.

A Blast search of the database yielded the identification of 31 different mammalian proteins that contain the FYVE domain (23). Currently, only a limited number of these proteins have been well characterized. KIAA0305 is an uncharacterized protein from Homo sapiens originally identified by the Kazusa DNA Research Institute (Chiba, Japan). A putative FYVE domain containing the characteristic conserved residues is present in the central region of KIAA0305, whereas its carboxy-terminal region is closely related to that of SARA, another FYVE domain protein. SARA is localized to vesicular structures in the cell, and it is thought to regulate Smad2 function by recruiting the latter molecule to the correct subcellular location in the cell, and it is thought to regulate Smad2 function. We propose that these two proteins may play a role in endosomal function.

**Expression Constructs**—The human cDNA of KIAA0305, which contains a predicted open reading frame of 1539 amino acid residues, was kindly provided by the Kazusa DNA Research Institute. KIAA0305 full-length and truncated cDNAs were cloned into the pDMycneo vector, which is a modified version of the pCIneo vector (Stratagene) with two MluI sites inserted 5' to the multiple cloning site. The full-length cDNA of SARA as well as that of Smad2 were obtained by PCR from a human brain Marathon cDNA library (CLONTECH) and subsequently cloned into the pDMycneo and pFLAG-CMV2 (Sigma-Aldrich) vectors, respectively. SARA with its coding sequence for the amino-terminal 665 amino acids deleted was also obtained by PCR and cloned into the pDMycneo vector. The constructs were confirmed by DNA sequencing.

**Antibodies**—The antigen, endofin (amino acids 1–250), was produced in bacteria as a GST fusion protein (GST305b). It was cleaved by thrombin and injected into rabbits with Freund’s adjuvant (Life Technologies, Inc.). Antiserum raised against endofin was purified using an affinity matrix prepared by chemically coupling the 250-amino acid immunogen to cyanogen bromide-Sepharose (Amersham Pharmacia Biotech). The antibody bound to the Sepharose was eluted with Immunopure IgG elution buffer (Pierce) and neutralized in 1 M Tris, pH 8. The antibody was then dialedyzed against phosphate-buffered saline, pH 7. Specific blocking of the antibody with its antigen was performed by incubating the antibody with 50-fold excess of GST305b for up to 4 h at 4 °C. Nonspecific blocking was similarly carried out in the presence of a GST fusion protein of the endofin putative Smad-binding domain (amino acid residues 814–962, GST305SSBD). The monoclonal antibody against EEA1 was purchased from Transduction Laboratories, and the anti-transferrin receptor hybridoma (OKT9) was obtained from American Type Culture Collection. Polyclonal anti-Myc antibodies for immunofluorescence analyses and immunoprecipitation were from Upstate Biotech Inc. and Santa Cruz, respectively. The monoclonal anti-HA and anti-FLAG antibodies were from Roche Molecular Biochemicals and Sigma-Aldrich, respectively. The secondary fluoroscein isothiocyanate (FITC), Texas Red-, or Cy3-conjugated anti-mouse or anti-rabbit antibodies were from Jackson ImmunoResearch.

**Immunofluorescence Analysis by Confocal Microscopy**—The cells were typically cultured on 18 × 18-mm glass coverslips and transiently transfected with the appropriate plasmids as mentioned in the text. All coverslips were washed with PBS containing 150 mM NaCl, 0.5% Triton X-100, and Complete EDTA-free protease inhibitors (Roche Molecular Biochemicals) and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature and followed by two washes in 50 mM ammonium chloride in PBS for 5 min each. Permeabilization was achieved by incubating the fixed cells in 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then blocked in FDB buffer (1 mM MgCl2, 1 mM CaCl2, 5% fetal calf serum, 5% goat serum, and 2% bovine serum albumin; BSA) in PBS for 30 min at room temperature. The cells were incubated with primary antibodies. All primary and secondary antibody incubations were performed in FDB for 1 h at room temperature. The cells were viewed using a confocal laser scanning microscope (Zeiss).

**Northern Blot Analysis**—A human multiple tissue Northern blot from CLONTECH Laboratories was hybridized with a 32P-labeled cDNA probe obtained by double digesting the KIAA0305 cDNA with SalI and BglII. The fragment used as a probe comprised the 5’-untranslated and -translated region (nucleotides 1–944 of KIAA0305). Hybridization and washes were carried out at 65 °C.

**Liposome Binding Assay**—Liposome binding assay and preparation of cytosols from A431 cells were performed as described previously (24). Phospholipids were purchased from the following sources: phosphatidylserine, phosphatidylinositol, and phosphatidylinositol 4-phosphate were from Sigma; phosphatidylinositol 3-phosphate and phosphatidylinositol 3,4-bisphosphate were from Matreya; and phosphatidylinositol 3,4,5-bisphosphate was from Roche Molecular Biochemicals. Western blotting for native EEA1 was carried out with a goat polyclonal anti-EEA1 antibody from Santa Cruz and detected with a peroxidase-conjugated anti-goat IgG antibody (Sigma).

**Immunoprecipitation and Western Blot**—Cells on 100-mm dishes were lysed in 0.5 ml of lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, and Complete EDTA-free protease inhibitor mixture (Roche Diagnostics GmbH). Immunoprecipitation was carried out at 4 °C with 4 μg of anti-Myc (Santa Cruz) or anti-HA (Roche Molecular Biochemicals) antibody in the presence of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) or protein G-agarose.
Endofin is a 230–250-kDa protein present in vesicular structures and diverse tissues. A, schematic representations of SARA and endofin comparing the organization of the FYVE domains (black boxes) and SBDs (striped boxes). The putative SBD of endofin is about 50% identical to that of SARA and 5 amino acids shorter. The amino-terminal coding sequence of endofin prior to the FYVE domain is divergent from that of SARA, whereas the carboxyl-terminal half after the FYVE domain is about 50% identical to SARA. The coding sequence for the first 250 amino acids of endofin was cloned into the pGEX vector and used as the immunogen (GST305b) for raising antibodies specific to endofin. The SBD region (amino acids 814–962) was also cloned into the pGEX vector for use as a negative control in the antibody blocking experiment. B, Western analysis of COS7 cells that were either transfected (+) or not transfected (−) with Myc-tagged endofin with antibody raised against endofin. The antibody was incubated with either GST305SBD as a negative control or with the immunogen GST305b, prior to probing the blot (panel a). COST cells that are either transfected (+) or not transfected (−) with Myc-tagged endofin were lysed, and the lysates were subjected to immunoprecipitation (IP) with either anti-Myc or anti-endofin antibody (panel b). The anti-endofin antibody is as effective as anti-Myc in immunoprecipitating Myc-tagged endofin. In addition, the antibody is able to immunoprecipitate endogenous endofin. The arrow indicates the 230–250-kDa band that represents both endogenous and Myc-tagged endofin. C, immunofluorescence analysis of A431 cells with anti-endofin antibody. When the antibody was preincubated with the immunogen, GST305b (panel b), Bar, 10 μm. D, Northern analysis of endofin expression. A Stul-BgIII 944-base pair fragment encompassing the 5′-untranslated region and amino-terminal coding sequence of KIAA0305 was used to probe a human multiple tissue Northern blot (CLONTECH). Two transcripts of 7.4 and 5.5 kilobases were detected.
acid sequences of endofin and SARA suggests a similar organization of the FYVE domain and a putative Smad-binding domain in endofin (Fig. 1A). Whereas the amino-terminal sequence of endofin is divergent from SARA, the carboxyl-terminal sequences of the two proteins starting from the FYVE domain onwards are strikingly similar, with up to 50% in sequence identity between the two proteins.

To study the biochemical properties of endofin, we raised polyclonal antibodies to the amino-terminal 250 amino acids of endofin. This region of endofin, which is divergent from SARA, was chosen so that the probability that the antibody will cross-react with the SARA protein is minimized. The antibody was purified and tested in both Western and indirect immunofluorescence analyses. The electrophoretic mobility of the endogenous protein in COS7 cells recognized by the anti-endofin antibody corresponded to a polypeptide of 230–250 kDa (Fig. 1B, panel a). A similarly sized band from COS7 cells overexpressing a Myc-tagged form of endofin was also recognized by the antibody. Anti-Myc antibody detected a band of the same size from cells transfected with Myc-tagged endofin (data not shown). To test the specificity of the anti-endofin antibody, Western blot analyses of cell lysates from both control and transfected COS7 cells were performed with the antibody first incubated with either the antigen or an irrelevant endofin fragment GST305SBD (residues 814–962; Fig. 1A). Neither endogenous or Myc-tagged endofin was detected when the antibody was preblocked with the antigen (Fig. 1B, panel a). In contrast, the 250-kDa band was detected when the antibody was preincubated with GST305SBD. These results suggest that the antibody is able to specifically recognize endofin. In addition, the antibody was also able to efficiently immunoprecipitate both endogenous and overexpressed endofin from COS7 cells (Fig. 1B, panel b). Smaller sized protein species that were also detected with the endofin antibody are likely to be proteolytic degradation products generated as a result of post-extraction handling of the cell extracts because these fragments were not as prominent in the lysates (Fig. 1B, panel b). Finally, the antibody was tested for its ability to detect the intracellular localization of endogenous endofin in A431 cells by immunofluorescence experiments. Indirect immunofluorescence analysis of these cells using anti-endofin preincubated with GST305SBD revealed the presence of endogenous endofin in punctate structures within the cells (Fig. 1C, panel a). In contrast, when the antibody was preincubated with the immunogen prior to incubation with the cells for immunodetection, no such signal was observed (Fig. 1C, panel b). These results establish that the observed punctate localization is specific for endofin.

Analysis of the tissue expression of endofin indicated that the gene is expressed in all tissues examined as a major RNA species of about 7.4 kilobases (Fig. 1D). A smaller transcript of about 5.5 kilobases, corresponding to the full-length endofin cDNA, was also detected. Thus, endofin appears to be a ubiquitously expressed protein.

Localization of Endofin to Early Endosomes—To determine the exact localization of endogenous endofin, we performed double immunolabeling of endofin with markers of the endosomal or secretory pathway. We found that endofin is enriched in early endosomes marked by EEA1 (Fig. 2). Endofin showed good co-localization with EEA1, suggesting that endofin resides in the early endosomal compartment (Fig. 2, panels a, a', and a''). To ascertain whether an epitope-tagged form of endofin could be faithfully targeted to the early endosome, cells were transfected with a construct for expressing endofin tagged with two Myc epitopes at its amino terminus. On immunostaining with anti-Myc antibody, the tagged protein, when expressed at low to moderate levels, appeared in vesicular structures similar to the endogenous protein and the staining pattern also overlaps with that of EEA1 (Fig. 2, panels b, b', and b''). The distribution of the exogenous epitope-tagged endofin, when expressed at low to moderate levels, is thus a reliable reflection of the endogenous protein.

The FYVE Domain Is Required for Endosomal Localization of Endofin—The FYVE domain has previously been shown to be important for the cellular localization of some members such as EEA1 (3) and SARA (15) but not for others such as Fgd1 (20) and Hrs-1 (22). To determine whether the FYVE domain is necessary for the intracellular localization of endofin, we made a series of expression constructs for truncated forms of the protein. The full-length or deletion constructs were expressed as Myc-tagged proteins, and their expression patterns were detected by immunofluorescence with anti-Myc antibody. When the amino-terminal 732 amino acids lacking the FYVE domain sequence were expressed, this endofin fragment appeared to be cytosolic (Fig. 3A). In contrast, when the first 855 amino acids, which includes the FYVE motif, were expressed, no apparent change in cellular location from the full-length endofin was observed (Fig. 3C). On the other hand, when the first 854 amino acids of the protein, which includes the FYVE domain, were deleted, the localization of the carboxyl-terminal fragment has changed from the vesicular pattern to a cytoplasmic one (Fig. 3D). However, when the FYVE domain was included in this truncated sequence, punctate vesicular staining...
FIG. 3. FYVE domain of endofin is required for endosomal localization. Schematic representations of the Myc-tagged deletion constructs of endofin for expression in A431 cells are shown on the left-hand side. The red boxes represent the FYVE domain. An asterisk represents the mutation of cysteine residue 753 to a serine residue (C753S). Numbers below the boxes indicate amino acid residue positions. Immunofluorescence data for the corresponding deletion constructs are shown on the right-hand side as indicated by the letters. The transfected cells were stained with anti-Myc antibody and visualized with Texas Red-labeled anti-rabbit IgG. Bar, 10 μm.

was again observed (Fig. 3c). By expressing only the central 100-amino acid region of the molecule containing the FYVE domain, we observed that the FYVE domain is sufficient to bring about a vesicular staining pattern (Fig. 3f). It is noted that the vesicular staining for the deletion constructs differed to varying extents from the fine punctate pattern observed previously for endogenous endofin (Figs. 1C and 2a). The vesicles appeared clustered or fused together into larger structures, an effect that was most pronounced when only the 100-amino acid FYVE domain region was expressed.

It has been shown previously for EEA1 that several conserved cysteine residues present in the FYVE domain are necessary for the localization of the protein (3, 25). To confirm the importance of the FYVE domain of endofin in determining its subcellular location, the first conserved cysteine residue at position 753 was mutated to a serine residue, and the expression pattern of the point mutant was detected as before. The C753S point mutant appeared to be cytosolic (Fig. 3g). Hence, an intact FYVE domain in endofin is essential and sufficient for determining the correct cellular localization of the protein.

Endosomal Localization of Endofin Is Dependent on PI 3-Kinase Activity—It has been previously demonstrated that wortmannin inhibits the endosomal association of EEA1 (24). Wortmannin is a potent inhibitor of the enzymatic activities of several mammalian PI 3-kinase isoforms (26, 27). To test the effect of wortmannin on endofin localization, we treated cells expressing Myc-tagged endofin with the drug followed by immunofluorescence detection with both anti-Myc and anti-EEA1 antibodies. Wortmannin treatment was found to cause a redistribution of both endofin and EEA1 from punctate vesicular structures to the cytosol (Fig. 4A). This result suggests that the cellular distribution of endofin is regulated by PI 3-kinase activity.

PI 3-kinase activity is required for the production of phospholipids phosphorylated at the 3′ position on the inositol head group. A prime example is PI3P. The ability of the FYVE fingers of EEA1 and Hrs-1 to bind specifically to PI3P has been well established (24, 28, 29). We therefore sought to determine whether the FYVE domain of endofin showed a similar specificity for binding to phospholipids. To address this issue, we carried out liposome binding experiments as described previously (24). Cytosols prepared from A431 cells transfected with the construct encoding for Myc-tagged endofin were incubated with liposomes containing a variety of phospholipids (Fig. 4B legend). The proteins bound to the liposomes were then resolved by SDS-PAGE and subjected to Western blot analyses with both anti-Myc and anti-EEA1 antibodies. The experiment revealed that Myc-tagged endofin bound very well to liposomes containing PI3P, somewhat less well to liposomes containing PI3,4P2, but not at all with liposomes composed of phospholipids not containing a 3′ phosphate group on the inositol ring (Fig. 4B, panel a). Endogenous EEA1, as detected by anti-EEA1 antibody, showed a similar binding profile, although its binding to PI3,4P2 seemed less intense in comparison with endofin (Fig. 4B, panel b). To determine whether an intact FYVE domain was required for PI3P binding, we also tested for the ability of the C753S endofin point mutant to bind to the various phospholipids in this assay. No binding of the C753S point mutant to PI3P-containing liposomes was observed (Fig. 4B, panel a). This result, together with the observation that an intact FYVE domain is required for the correct localization of endofin (see above), suggests that the inability of the C753S mutant to localize correctly is due to its inability to bind to PI3P and/or to a lesser extent PI3,4P2 present in the early endosomes.

Endofin Co-localizes with SARA but Does Not Associate with It or Smad2 and Does Not Behave Like SARA in Affecting TGF-β Signaling—Because endofin is structurally related to SARA, it is of interest to determine the subcellular relationship of these two proteins. We first examined the intracellular localizations of both endofin and SARA by co-expressing endofin as an HA-tagged protein and SARA as a Myc-tagged protein in the Mv1Lu mink lung epithelial cell line. Indirect immunofluorescence analysis using both anti-HA and anti-Myc antibodies
revealed that endofin and SARA co-localized well in intracellular vesicular structures (Fig. 5A). This observation prompted us to examine the biochemical relationship between the two proteins. Several FYVE domain proteins have been shown to exist as complexes. For instance, EEA1 is found to be associated with a number of molecules involved in endocytosis such as Rab5 and syntaxins 6 and 13 (5, 30, 31), whereas Hrs-1 is associated with STAM and Smads (13, 14). Moreover, EEA1 has been shown to be able to self-associate into a dimer that may facilitate its role as a tethering molecule in endocytosis (32). We therefore proceeded to analyze the ability of endofin to associate with itself or with SARA. For this purpose, Myc-tagged endofin was co-expressed with its HA-tagged form and immunoprecipitated with either anti-Myc or anti-HA antibody. Neither the anti-Myc or anti-HA antibody was found to be effective in pulling down the HA-tagged or Myc-tagged endofin, respectively (Fig. 5B). This suggests that endofin does not associate with itself. We performed the same experiment with Myc-tagged SARA and HA-tagged endofin co-expressed in COS7 cells (Fig. 5B). As before, we were unable to detect the co-immunoprecipitation of SARA with endofin, suggesting that these two proteins are not associated with each other. We were similarly unable to co-immunoprecipitate endogenous EEA1 with Myc-tagged endofin (data not shown).

The ability of SARA to affect TGF-β signaling via interaction with Smads is an important property attributed to the SBD of the molecule (15). Because endofin also contains a putative SBD similar to that in SARA, we sought to determine whether endofin is able to associate with Smad. To this end, we co-transfected 293T cells with vectors for expression of FLAG-tagged Smad2 and either Myc-tagged SARA or endofin. Immunoprecipitation analysis with anti-Myc antibody indicated that Myc-tagged SARA is able to bind to Smad2 efficiently (Fig. 5C), as reported previously (15). In sharp contrast, Myc-tagged endofin was unable to do so (Fig. 5C). This result suggests that the SBD in endofin may either be inactive toward Smad2 binding or functions very inefficiently relative to that in SARA.

We next tested the ability of endofin or its truncated mutant to disrupt TGF-β-induced transcription. Although full-length SARA has no effect on TGF-β signaling, SARA with its first 665 amino acids deleted (SARAΔ1–665) has been previously shown to behave as a dominant negative mutant that significantly inhibited TGF-β-dependent responsiveness of transfected cells (15). An endofin mutant analogous to SARAΔ1–665 (with its amino-terminal 814 amino acids deleted, endofinΔ1–814) was thus constructed and used for comparison. The experiment was carried out with Mv1Lu cells transiently transfected with the TGF-β-responsive reporter gene 3TP-lux in the presence of either full-length SARA, endofin, or their truncated versions. As predicted, expression of either full-length SARA or endofin had no effect on TGF-β-induced transcriptional activity (Fig. 5D). Expression of SARAΔ1–665 caused an inhibition in TGF-β responsiveness in a manner that is dependent on the amount of DNA transfected, as described previously (15). In contrast, transfection of endofinΔ1–814, even at high concentration, had no effect on TGF-β-dependent signaling. These results suggest that, unlike SARA, endofin may be not involved in the TGF-β signaling pathway.

**Overexpression of Endofin Causes Endosomal Aggregation**—We observed previously that overexpression of truncated endofin mutants resulted in aggregated or fused vesicular structures (see above). To investigate this issue further, we examined the effect on endosomes when full-length endofin was overexpressed in A431 cells. These cells were transfected with the construct encoding the Myc-tagged protein, and the effect was traced by labeling the cells with EEA1 for early endosomes and transferrin receptor for recycling endosomes. At high levels of expression, endofin resulted in a vesicular aggregation or fusion effect (Fig. 6). These aggregated vesicles contained both EEA1 (Fig. 6A, panels a, a′, and a″) and transferrin receptor (Fig. 6A, panels b, b′, and b″), indicating that they are derived from early or recycling endosomes or both. Like its truncated counterparts, this experiment shows that full-length endofin is also able to bring about endosomal aggregation or fusion.

Because both endofin and SARA are structurally related proteins that appear to be located in the same endosomal structures, the next question is whether they have a similar effect on endosome morphology. For this purpose, we used COS7 cells because this cell line can support higher expression levels of exogenous proteins. COS7 cells were transfected with plasmids that express HA-tagged endofin or Myc-tagged SARA or with both plasmids simultaneously. The cells were then subjected to immunofluorescence analysis as described before. At low to moderate levels of expression, the staining pattern for HA-tagged endofin resembled that of normal endosomes (Fig. 6B, panel a). At high levels of expression, endocytic aggregates/fusions (Fig. 6B, panel b) or enlarged vesicular structures of up to about 5 μm in diameter can be observed (Fig. 6B, panel c). It
is clear from the enlarged vesicles that the staining decorates only the peripheral rim of the structure and not the interior, in agreement with the notion that the FYVE domain is associated with the cytoplasmic side of membrane PI3P and/or PI3,4P₂. When HA-endofin was highly overexpressed, endocytic aggregates/fusions accounted for 90% of the abnormal vesicular structures observed, whereas the remaining 10% of high expressers showed enlarged vesicles. Thus, high level endofin expression has the tendency to cause endosomal aggregation/fusion. A similar dynamic organization of immunopositive vesicular structures was observed when Myc-tagged SARA was overexpressed alone (Fig. 6B, panels d–f). There are, however, some distinct differences. First of all, much more dramatic enlargement of endocytic structures can be observed (Fig. 6B, panel f). These vesicles can reach sizes that are 10 times larger than normal endosomes (up to 9 μm in diameter), a phenomenon not observed for HA-endofin. Secondly, the proportion of cells highly overexpressing SARA that contained enlarged endocytic vesicles was at least three times that observed when HA-endofin was overexpressed alone. Thus, high expression of SARA causes more dramatic changes in endosomal size more frequently than endofin. When both proteins were overexpressed in COS7 cells, the frequencies of endosome aggregation/fusion and enlargement effects were only slightly moderated by the presence of both proteins (Fig. 6B, panels g, g', g'', h, h', h'', i, i', and i''). These experiments show that endofin and SARA can regulate endosomal morphology.

**Accumulation of EGF in Cells Overexpressing Endofin or Its Membrane-associated Truncated Mutants**—We next investigated the functional properties of the endosomal aggregates/fusions caused by high expression levels of endofin and its truncated forms. We examined the ability of these endosome clusters to participate in transport by monitoring the endocytosis of EGF. The events of the EGF receptor endocytosis pathway have been well mapped out. Binding of EGF to its receptor induces the ligand-receptor complex to be recruited to clathrin-coated pits followed by internalization to the early endosome where it is either recycled back to the cell surface or delivered to the late endosome and subsequently lysosomes for degradation. The latter pathway is the predominant event and represents the process whereby EGF signaling is attenuated. Disruption in transport to the lysosomes results in the inhibition of EGF breakdown (33). A431 cells transfected with full-length endofin and its membrane-associated truncated forms (Fig. 3, c, e, and f) were preincubated with Oregon green-labeled EGF for 1 h on ice followed by the initiation of endocytosis at 37 °C. Labeled EGF uptake was analyzed 2 h post-initiation. The EGF fluorescence at the end of this incubation period was significantly low in most untransfected cells due to proteolytic deg-
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Fig. 6. High levels of expression of endofin cause endosome aggregation. A, immunofluorescence analysis of A431 cells overexpressing Myc-tagged endofin. The cells were fixed, permeabilized, and stained with anti-Myc (panel a) and anti-EEA1 antibodies (panel a’ or anti-Myc (panel b) and anti-transferrin receptor (TfR) antibodies (panel b’). Each row represents the same field, and merged images are shown in the third column as overlays (panels a’ and b’). The polyclonal anti-Myc was visualized with Texas red-conjugated anti-rabbit IgG, whereas the anti-EEA1 and anti-transferrin receptor antibody stainings were visualized with FITC-conjugated anti-mouse IgG. Yellow represents areas of overlapping labeling. Bar, 10 μm. B, a panel of confocal micrographs showing immunolocalization of HA-tagged endofin, Myc-tagged SARA, or both HA-tagged endofin and Myc-tagged SARA simultaneously transfected in COS7 cells. In cells transfected with HA-tagged endofin alone, anti-HA immunolabeling was detected in punctate vesicular structures of regular size in cells presenting low to moderate expression levels (panel a), in both vesicular structures and aggregates/fusions (panel b), or in vesicles of up to five times the regular size (panel c) when expressed at high levels. In cells transfected with Myc-tagged SARA alone, anti-Myc immunolabeling was detected in punctate vesicles of regular size in cells expressing the protein at low to moderate levels (panel d), in both vesicles and aggregates/fusions (panel e) or in vesicles exhibiting great heterogeneity in size (panel f) when present at high levels. Very large vesicles, up to 10 times the size of regular vesicular structures, can be observed. In cells simultaneously expressing both HA-tagged endofin and Myc-tagged SARA, immunolabeling with anti-HA (panels g-i) and anti-Myc antibodies (panel g’, h’, i’, and f’) also detected the proteins in punctate vesicular structures of regular size at low to moderate expression levels (panels g’ and g’), whereas at high expression levels, aggregates/fusions (panels h, h’, i, and f’) can be observed. Each row (panels g, g’, h, h’, i, i’, and f’) represents the same field and, merged images are shown in the third column as overlays (panels g’, h’, i’, and f’). Anti-HA antibody was detected with Cy3-labeled anti-mouse IgG, and anti-Myc antibody was detected with FITC-labeled anti-rabbit IgG. Yellow represents regions of overlapping labeling. Bar, 15 μm.

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SARA was earlier identified as a FYVE domain protein important in TGF-β signal transduction (15). It has a domain structure and carboxyl-terminal sequences that are similar to endofin. A similar domain architecture has also been reported for a Caenorhabditis elegans protein kinase A anchoring protein (AKAP(C3)), identified by sequence-based bioinformatics methods (36). This molecule, however, does not contain a potential SBD, and it is unclear whether it has a functional FYVE domain because the second conserved cysteine residue is missing in the sequence. Nonetheless, based on the sequence similarities, these two molecules may form a subfamily of SARA-like proteins. We report here that endofin and SARA co-localize well in endosomal structures in the cell. This observation suggests that endofin and SARA may both function in the early endosome. We then addressed the question as to whether they cooperate with each other in a functional complex. Co-immunoprecipitation experiments using cells co-expressing both proteins revealed that endofin and SARA are not associated with each other, suggesting that the two proteins are likely to be found in separate complexes in the same endosomal compartment. Furthermore, endofin is unable to interact with Smad2 or to participate in TGF-β signaling in a manner similar to SARA, implying strongly that endofin and SARA are not functionally redundant molecules. Additional work is required to address the issue regarding the ability of endofin to associate with other Smad members and its potential role in other signaling pathways.

Overexpression of several FYVE domain proteins has been reported to lead to changes in endosomal structure. Hrs-1, when overexpressed in HeLa cells, resulted in early endosomes that appear larger than normal or aggregated (35, 37). Rabip4 overexpression in CHO cells has also been reported to lead to an increase in the size of early endosomes (9). We describe a similar observation when endofin was overexpressed at high levels in both A431 and COS7 cells. The aggregated/fused or enlarged vesicular structures represent the early/recycling endocytic compartment because they contain both EEA1 and transferrin receptor, markers for the early and recycling endosomes, respectively. This is an interesting observation because early/recycling endosome expansion is associated with increased endosome fusion activity, such as that contributed by the active GTP-bound form of Rab5 (38) or by overexpression of wild-type Rab5 (34). Indeed, both Hrs-1 and Rabip4 have been implicated in early endosome function (9, 39). This suggests that endofin may also be involved in endosome fusion during endocytosis.

The morphological changes in endosomes associated with endofin or SARA overexpression are distinctly profound in COS7 cells, which allow higher levels of expression of exogenous proteins. Although both endofin and SARA appear to cause mainly endosomal aggregation/fusion effects, there are differences in the frequency and nature of the endosome expansion effect. Endofin overexpression appears to cause a higher frequency of endosomal aggregation/fusion than SARA, whereas SARA overexpression seems to result in an endosomal enlargement effect that is more dramatic and in higher frequency than those observed for endofin. Simultaneous overexpression of both proteins did not significantly change the frequency of aggregate/fusion or vesicular enlargement events in comparison with overexpression of either protein alone. This suggests that the two proteins do not cooperate synergistically or additively with each other to cause aggregated/fused or enlarged endosomes. Our observations therefore raise the possibility that endofin and SARA are involved in endosome fusion activity but that they do so in separate and unique ways. These differential effects may be conferred by the amino-terminal regions of the two molecules because these are divergent from each other.

The alterations in endosome morphology caused by overexpression of endofin may be attributed to the endofin FYVE domain because expression of this region alone is sufficient to cause the endosomal aggregation/fusion effects. This property of the endofin FYVE domain is likely to be unique and not common to other FYVE domains. For a start, as mentioned before, the FYVE domain of EEA1 by itself was insufficient to localize to endosome. Secondly, the FYVE domain of Hrs-1 is cytosolic when expressed alone (22). We also report that these aggregated endocytic structures represent dysfunctional endosomes because endocytosed EGF was retained and prevented from transport to lysosomes for proteolytic degradation. The amino- and carboxyl-terminal regions of endofin lacking the FYVE domain did not inhibit EGF degradation in the same experiment (data not shown). These findings, coupled with the observed differential effects resulting from overexpression of either endofin or SARA, suggest that although the endofin FYVE domain alone is sufficient to participate in endosome function, the actual role of endofin in the early endosomes may be moderated by other regions of the molecule. Further work is...
therefore required to discover the structural and molecular components involved in causing the differential endosomal effects contributed by either endofin or SARA.

In summary, we report here that endofin is a ubiquitous protein present in the early endosomes and that its endosomal association is mediated by its FYVE domain. Although significant differences were observed with regards to the alteration of endosomal morphology resulting from overexpression of either endofin or SARA, the similar structure, endosomal localization, and observed alterations on endosomal compartment upon overexpression suggest that both molecules may be involved in endosome function. Future work involves addressing the specific role and molecular targets of endofin in the endocytic pathway.

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