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Endofin Recruits TOM1 to Endosomes*

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Endofin is an endosomal protein implicated in regulating membrane trafficking. It is characterized by the presence of a phosphatidylinositol 3-phosphate-binding FYVE domain positioned in the middle of the molecule. To determine its potential effectors or binding partners, we used the carboxyl-terminal half of endofin as bait to screen a human brain cDNA library in a yeast two-hybrid system. Three clones that encode TOM1 were recovered. TOM1 is a protein closely related to the VHS (VPS-27, Hrs, and STAM) domain-containing GGA family. Although the function of the GGAs in mediating Golgi to lysosomal trafficking is well established, the subcellular localization and function of TOM1 remain unknown. Glutathione *S*-transferase pull-down assays as well as co-immunoprecipitation experiments confirmed that the carboxyl-terminal half of endofin binds specifically to the carboxyl-terminal region of TOM1. Neither SARA nor Hrs, two other FYVE domain proteins, interact with this region of TOM1. Moreover, endofin does not interact with the analogous region of two other members of the TOM1 protein family, namely, TOM1-like 1 (TOM1-L1) or TOM1-like 2 (TOM1-L2). The carboxyl-terminal region of TOM1 was used as immunogen to generate TOM1-specific antibody. This antibody can distinguish TOM1 from the other family members as well as co-immunoprecipitate endogenous endofin. It also revealed the primarily cytosolic distribution of TOM1 in a variety of cell types by immunofluorescence analyses. In addition, sucrose density gradient analysis showed that both TOM1 and endofin can be detected in cellular compartments marked by the early endosomal marker EEA1. A marked recruitment of TOM1 to endosomes was observed in cells overexpressing endofin or its carboxyl-terminal fragment, indicating TOM1 to be an effector for endofin and suggesting a possible role for TOM1 in endosomal trafficking.

Endofin is a member of the FYVE domain family of proteins. The FYVE finger domain (1) is involved in interaction with the phospholipid phosphatidylinositol 3-phosphate, which is found in significant levels on endosomal membranes (2). In many instances, therefore, the FYVE domain serves to localize the protein containing it to endosomes. In endofin, the FYVE domain appears to be necessary and sufficient to localize the protein to early endosomes (3). The FYVE domain is a modular motif that can function independent of its position on the molecular structure. In the case of endofin, the FYVE domain sits in the middle of the molecule, thereby effectively dividing the molecule into two halves.

Apart from a few exceptions like the proteins FGD1 (faciogenital dysplasia gene product 1) (4), Frabin (5), and DFCP1 (double FYVE-containing protein 1) (6), members of the mammalian FYVE domain family have, in general, two characteristics in common: the presence of the FYVE domain and the consequential location of the proteins on endosomal membranes. The presence of these proteins on endosomal structures imply that they have roles in trafficking and/or signaling involving endosomes. The nature of their specific roles is thought to be dependent on the structural information encoded by the sequences of the proteins outside of the FYVE domain. These sequences may encode for catalytic activities as in PIKfyve (phosphoinositide kinase with specificity for five position containing a fyve finger), which is a phosphoinositide 5-kinase (7), and FYVE-DSP1 and 2 (FYVE dual-specificity protein phosphatases 1 and 2), which are dual specificity protein phosphatases that can also dephosphorylate phosphatidylinositol 3-phosphate (8, 9).

The FYVE domain flanking or adjacent sequences may also represent binding sites for downstream effector molecules or binding partners. In these cases, identifying the interacting partners is crucial to an understanding of the specific functions of individual FYVE domain proteins. To this end, the functions of a few members of the FYVE domain protein family are becoming clearer because of the identification of their interacting partners. The prototype FYVE domain protein, EEA1 (early endosome autoantigen 1) (10) is the most extensively characterized. EEA1 consists of an amino-terminal C_2H_2 Zn^{2+} finger, four heptad repeats, and a carboxyl-terminal region containing a calmodulin-binding (IQ) motif and a FYVE domain. EEA1 has been shown to bind to Rab22 through its amino-terminal region (11) and is known to associate with Rab5 through both the amino-terminal region as well as an additional binding site in its carboxyl-terminal region (12). The IQ domain has been shown to be required for binding to calmodulin (13). In addition, it has been demonstrated to interact with two SNAREs,[1] Syntaxin 6 and Syntaxin 13, at its carboxyl terminus (13–15). The discovery of the association between EEA1 and multiple proteins involved in endosome fusion has thus cemented the importance of this molecule in regulating the endosome fusion pathway (16). Another well characterized FYVE domain protein is the Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (17). Like EEA1, Hrs contains multiple domains: an amino-terminal VHS domain, a FYVE domain, a coiled-coil domain, and a clathrin-binding domain. The amino-

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[1] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; GST, glutathione *S*-transferase; HA, hemagglutinin.

terminal region before the coiled-coil domain is found to bind to Eps15, a protein required for receptor-mediated endocytosis via interactions with α-adaptin (18). The Hrs coiled-coil domain and the carboxyl-terminal region have in turn been shown to bind to a variety of molecules: STAM1 and STAM2, which are downstream effectors of the Janus kinases in cytokine signaling (19); the SNARE protein SNAP-25, which is a component of the complexes involved in vesicle docking and fusion (20); the Smad proteins, which are effector molecules that transmit signals from the transforming growth factor receptor β to the nucleus (21); and SNX1 (sorting nexin 1), which is involved in lysosomal trafficking (22). The clathrin-binding domain of Hrs is functional and has been reported to interact directly with the clathrin heavy chain (23). In addition, Hrs contains a ubiquitin-interacting motif where it binds directly to ubiquitin (24, 25) as well as a PSAP motif that facilitates interaction with the ESCRT-I subunit Tsg101 (26–28). From the identification of the multiple interacting partners for Hrs, it can be envisioned that the protein is likely to bridge signal transduction and regulation of the trafficking event that sorts ubiquitinated proteins from endosomes to the degradative pathway (29). Similarly, SARA, another FYVE domain-containing protein, is likely to be involved in both signaling events and intracellular trafficking. SARA contains a binding site known as the Smad-binding domain that interacts with both Smad2 and Smad3 (30). Downstream transforming growth factor receptor β signal transduction is not only dependent on this interaction, it also requires that SARA is properly localized on endosomes (31) and that endosome function was not disrupted (32). Other FYVE domain proteins that have been ascribed specific roles based on their interacting partners have also been described. Rabenosyn5 interacts directly with Rab5 and hence, like EEA1, is a component of the Rab5 effector complex(es) (33). The mouse FYVE domain protein Rabip4 (Rab 4-interacting protein) was originally isolated as a downstream effector of Rab4, a small GTPase implicated in regulating endocytosis (34). More recently, the human ortholog of Rabip4, designated RUFY1 (RUN and FYVE-domain-containing protein 1), has been reported to bind to epithelial and endothelial tyrosine kinase, a cytoplasmic tyrosine kinase. This interaction suggests again the coupling of endocytosis and the regulation of cellular processes such as proliferation, differentiation, cell motility, and apoptosis (35).

In this report, we describe the identification of an endofin binding protein, TOM1, from a yeast two-hybrid screen. We demonstrate the interaction of endofin with TOM1 by both *in vitro* and *in vivo* methods. We also show that endofin interacts with TOM1 via its carboxyl-terminal half. Coincidentally, it is the carboxyl-terminal region of TOM1 that is responsible for binding to endofin. We provide evidence that although TOM1 appears to be a cytoplasmic protein by immunofluorescence analysis, its distribution does partially co-localize with that of endofin by density gradient analysis. Overexpression of endofin has a significant impact on TOM1 localization as we show that endofin can cause a shift of TOM1 distribution onto the endosomes. These data provide evidence for a role of endofin in regulating the distribution of TOM1 as well as the potential involvement of TOM1 in endosomal function.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The full-length cDNAs of human TOM1, TOM1-like 1 (TOM1-L1) and TOM1-like 2 (TOM1-L2), were cloned by PCR from a human fetal brain Marathon cDNA library (Clontech), whereas the Hrs cDNA was cloned from a human kidney Marathon cDNA library (Clontech). The full-length TOM1 cDNA was subsequently cloned into the vector pFLAG-CMV2 (Sigma-Aldrich), behind the FLAG peptide sequence, for transient expression in mammalian cells. The truncated TOM1, TOM1-L1, or TOM-L2 constructs were also generated by PCR and cloned into the pGEX-4T vector (Amersham Biosciences) for expression in *Escherichia coli*. The cDNA encoding the endofin fragment from amino acids 815–1539 was generated by PCR and cloned into the pDMycneo vector as for the other endofin constructs described previously (3).

Yeast Two-hybrid Screen—The cDNA coding for the carboxyl-terminal half of endofin from amino acids 806–1539 was generated by PCR and cloned into the vector pGBKT7. The resulting GAL4-endofin (806–1539) fusion construct was used as a bait for screening a human brain cDNA Matchmaker GAL4 yeast two-hybrid library (Clontech) according to the manufacturer's instructions.

Cell Culture and Transient Expression—293T, A431, HeLa, BHK21, Chinese hamster ovary, and NRK cells were grown as described previously (3). Transfection of the cells for transient expression was carried out using LipofectAMINE 2000 (Invitrogen) for about 5 h according to the manufacturer's instructions. The cells were then processed for subsequent GST pull-down assays, immunoprecipitation experiments, or immunofluorescence analysis 48 h post-transfection.

Immunoprecipitation and Western Blot—The cells were lysed in 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 Applied Science). Immunoprecipitation followed by Western blotting were carried out as described previously (3). For co-immunoprecipitation of endogenous TOM1 and endofin, A431 cell lysate was incubated with 20 μg of either anti-TOM1 or anti-endofin antibody as well as 20 μl (bed volume) of protein A-Sepharose CL-4B (Amersham Biosciences) at 4 °C overnight. After washing several times with lysis buffer, bound proteins were analyzed as before (13).

GST Pull-down Assays—GST and various GST fusion proteins were produced in *E. coli* according to procedures described for the glutathione *S*-transferase Gene Fusion System (Amersham Biosciences). GST or GST fusion protein was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 2–4 h at 4 °C followed by extensive washing in phosphate-buffered saline. An aliquot containing 20 μg of GST or GST fusion protein bound to beads was then incubated with cell lysates prepared as described above for immunoprecipitation analysis. After 4 h at 4 °C, the beads were washed three times with lysis buffer. The bound proteins were eluted by incubating the beads in SDS loading buffer containing dithiothreitol at 95 °C for 5 min. Electrophoresis followed by Western blotting were carried out as described previously (3).

Antibodies—Antibodies specific for TOM1 were produced in rabbits by injecting bacterially produced recombinant TOM1 fragment (amino acids 300–492) coupled to GST as a fusion protein. Antiserum so raised was purified using an affinity matrix prepared by chemically coupling the antigen to glutathione-Sepharose using dimethyl pimelimidate dihydrochlorite (Sigma). Antibody bound to the Sepharose was eluted with ImmunoPure IgG elution buffer (Pierce) and neutralized in 1 M Tris-HCl, pH 8. The antibody was then dialyzed against phosphate-buffered saline, pH 7, and concentrated using Centricon (Millipore). Specific blocking of the antibody with its antigen was achieved by incubating the antibody with a 50-fold excess of GST-TOM1(300–492) for at least 4 h at 4 °C. Nonspecific blocking was similarly carried out with either an irrelevant GSTTOM1 fusion (GST-TOM1(2–152)) or GST-TOM1-L1(285–467).

The following antibodies were obtained from the indicated commercial sources: polyclonal anti-Myc tag for Western blotting and immunofluorescence studies from Upstate Biotech Inc.; polyclonal anti-Myc tag for immunoprecipitation experiments from Santa Cruz; monoclonal anti-FLAG tag from Roche Applied Science; polyclonal anti-GST from Amersham Biosciences; and monoclonal anti-EEA1 from Transduction Laboratories. The rabbit anti-endofin antibody was obtained as described previously (3). The secondary fluorescein isothiocyanate- or Cy3-conjugated anti-mouse and anti-rabbit antibodies were from Jackson ImmunoResearch.

Immunofluorescence Analysis by Confocal Microscopy—A431 cells were fixed, permeabilized, and stained as described previously (3). The cells were viewed and the images taken using a confocal laser scanning microscope (MRC1024; Zeiss).

Sucrose Gradients—The procedure was adapted from a previous report (36). A431 cells were washed twice in phosphate-buffered saline followed by single wash in cytosol buffer (0.2 M sucrose, 25 mM Hepes, pH 7.3, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). The cell monolayers were scraped off into residual cytosol buffer remaining on the dish and disrupted by 40 strokes through a 26-gauge needle. Nuclei and unbroken cells were removed by centrifugation at 500 × g for 5 min. The postnuclear supernatant was then loaded on top of a 4-ml column

of 10–40% discontinuous sucrose gradient in 25 mM Hepes, pH 7.3. The column was spun at 40, 000 rpm for at least 16 h in a Beckmann L7–55 ultracentrifuge using the SW60 Ti swinging bucket rotor. The tube was punctured at the bottom with an 18 gauge needle, and 250-μl fractions were collected into Eppendorf microcentrifuges containing 2× SDS loading buffer. A small aliquot of the postnuclear supernatant as well as a 25-μl aliquot of each fraction were analyzed by SDS-PAGE and Western blotting.

RESULTS

Identification of TOM1 as an Endofin-binding Protein—To isolate proteins that interact with endofin, a yeast two-hybrid screen was performed using the carboxyl-terminal half of human endofin as bait. Specifically, a GAL4-endofin (806–1539) fusion construct was used to screen a human brain cDNA library (Clontech) (Fig. 1*A*). 253 positive transformants were sequenced to determine the identity of the clones. Interestingly, three of the transformants contained sequences encoding for human TOM1 (34). The interaction of endofin with TOM1 was subjected to further study, and the results are presented below.

TOM1 was first identified as a member of a superfamily of proteins that features the VHS domain (38). In addition to the amino-terminal VHS domain of about 150 amino acids that includes a single NPF motif, TOM1 contains a central GAT domain that is so named because it has some sequence homology to GGA (*G*GA *a*nd *T*OM1) (Fig. 1*B*). To confirm the yeast two-hybrid interaction mentioned above, we cloned the full-length TOM1 sequence from human fetal brain by PCR. Attempts were then made to produce the GST-conjugated form of TOM1 protein in *E. coli*, but great difficulty was encountered in doing so. As such, we designed constructs that express the individual domains of TOM1 instead. The VHS domain (amino acids 2–152), GAT domain (amino acids 153–299), and the remaining carboxyl-terminal sequence (amino acids 300–492) of TOM1 fused to GST can be successfully expressed in bacteria.

To establish an interaction between endofin and TOM1 via alternative approaches, we examined the association between endofin and TOM1 in a series of GST pull-down assays. Myc-tagged endofin deleted of the first 814 amino acids (Myc-Endofin(815–1539)), which is similar to the bait used in the yeast two-hybrid screen, was first examined for its ability to bind to the various GST-fused TOM1 domains. Whole cell lysate prepared from 293T cells transfected with the plasmid encoding for Myc-tagged Endofin(815–1539) was incubated with each of the GST-TOM1 domains bound to glutathione resins. Proteins bound to the resin were then resolved by SDS-PAGE followed by Western blotting and probed with anti-Myc antibody. As shown in Fig. 1*C* (*panel ii*), Myc-tagged Endofin(815–1539) binds only to the carboxyl terminus of TOM1 but not to GST alone nor to the VHS or GAT domains of TOM1. This result suggests that endofin binds specifically to the carboxyl terminus of TOM1, which has a unique sequence in comparison with other members of the VHS/GGA family. The same experiment was repeated with lysates from 293T cells transfected with either Myc-tagged full-length endofin or endofin deleted of 807 of its carboxyl-terminal amino acids (Myc-Endofin(1–732)). Full-length endofin was found to bind specifically to the carboxyl terminus of TOM1 in a manner similar to that of Myc-Endofin(815–1539) (Fig. 1*C*, *panel i*). In contrast, Myc-Endofin(1–732) cannot bind to any domain of TOM1 (Fig. 1*C*, *panel iii*). These results indicate that the binding site(s) for TOM1 resides only in the carboxyl-terminal half of endofin and that endofin interacts specifically with the carboxyl-terminal unique region of TOM1.

To further define the endofin-binding site on TOM1, we constructed more TOM1 mutants expressing progressively shorter carboxyl-terminal sequences as fusion proteins with GST. In pull-down assays similar to those described above, overexpressed Myc-tagged endofin was able to interact with the TOM1 carboxyl-terminal region from amino acids 322 to 492 but not with the shorter sequence from amino acids 353 to 492 (Fig. 1*D*). Thus, the binding domain for endofin on TOM1 involves critical residues spanning the region from amino acids 322 to 353.

The interaction between endofin and TOM1 *in vivo* was next examined. Plasmids encoding the various Myc-tagged forms of endofin were designed (Fig. 2*A*, *left panel*, and Ref. 3). Each of these plasmids was co-transfected with a plasmid encoding FLAG epitope-tagged full-length TOM1 into 293T cells. Whole cell lysates of the transiently transfected cells were prepared and subjected to immunoprecipitation with anti-Myc antibody followed by SDS-PAGE and Western blotting with anti-FLAG antibody. When full-length endofin is co-expressed with TOM1, it is able to co-immunoprecipitate with the latter (Fig. 2*A*, *right panel*). This property is unaltered even when the FYVE domain was mutated (EndofinC753S). We have shown previously that this mutant is unable to bind phosphatidylinositol 3-phosphate and does not have an endosomal distribution (3). This suggests that the endosomal localization of endofin is not essential for the interaction between endofin and TOM1. In contrast, no co-immunoprecipitation of TOM1 was observed for the two endofin mutants deleted of the carboxyl terminus (Myc-Endofin-(1–962) and Myc-Endofin(1–732)). The failure of these two carboxyl-terminally truncated mutants to bind to TOM1 confirms the above GST pull-down data. On the other hand, the carboxyl-terminal half of endofin, with or without the FYVE domain (Myc-Endofin(732–1539) and Myc-Endofin(815–1539), respectively), can interact with TOM1. Again, the presence or lack of the FYVE domain does not interfere with the ability of the carboxyl-terminal sequence of endofin to interact with TOM1. These data corroborate to indicate that the carboxyl-terminal half of endofin contains a binding site for TOM1 that functions independently of the FYVE domain.

To further delineate the TOM1-binding site on endofin, we tested the ability of various endofin carboxyl-terminal deletion constructs to interact with the GST-fused TOM1 carboxyl-terminal fragment (amino acids 300–492) (Fig. 2*B*, *left panel*). In pull-down experiments similar to those described previously, it was found that GST-TOM1(300–492) was able to associate with endofin encoding amino acids 1001–1539 but not with the shorter fragment encoding from amino acid residue 1114 (Fig. 2*B*, *right panel*). This indicates that the stretch of amino acids from positions 1001 to 1114 contains the residues critical for interaction with TOM1. Surprisingly, these residues, on their own, are insufficient for the interaction because the endofin fragment containing this region but lacking the carboxyl-terminal 20 amino acids (Myc-Endofin(1001–1519)) was unable to interact with the TOM1 fusion protein. This indicates that the TOM1-binding domain on endofin encompasses at least two regions: from residues 1001 to 1114 as well as the carboxyl-terminal 20 amino acids. The involvement of at least two discontinuous regions suggests that conformational information of the carboxyl-terminal region of endofin is likely the basis for interaction with the unique sequence of TOM1.

TOM1 C-terminal Sequence Associates Specifically with Endofin but Not SARA or Hrs—The association of TOM1 with endofin prompted us to examine the possibility that this protein may also interact with other FYVE domain proteins. To this end, we tested the ability of two other members, namely SARA and Hrs, to associate with the carboxyl terminus of TOM1 by GST fusion pull-down analysis as described before. In this instance, Myc-tagged endofin, which was used as a positive

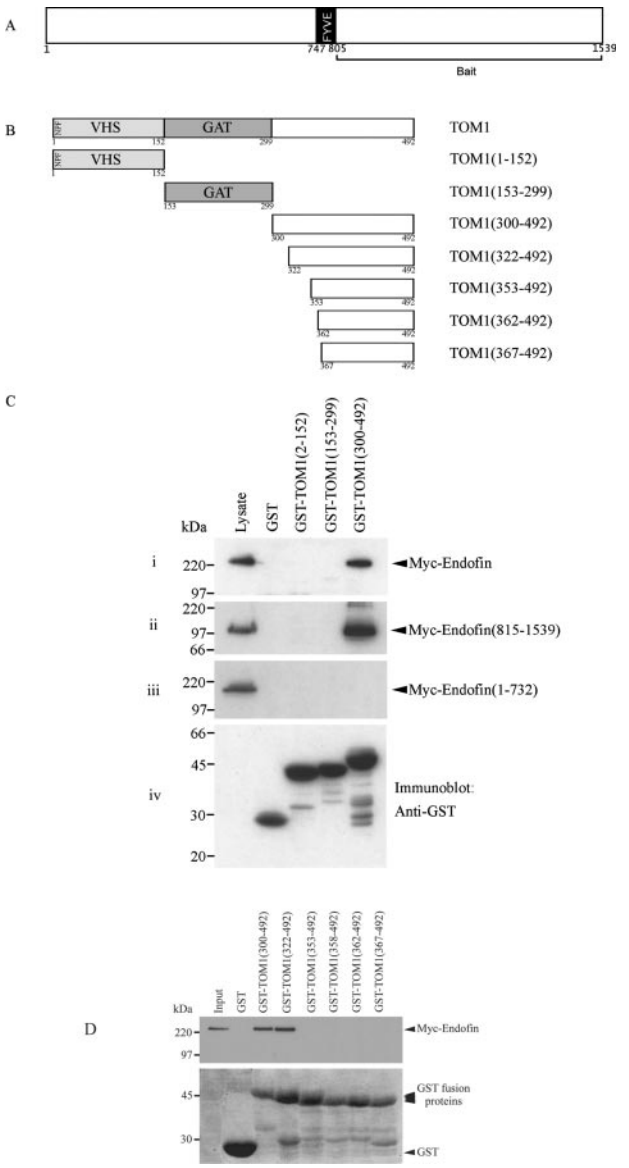

FIG. 1. **Endofin binds to the carboxyl-terminal region of TOM1 via its carboxyl-terminal sequence.** *A*, schematic representation of endofin and the region used as the bait in yeast two-hybrid screen for interacting proteins. The FYVE domain (*black box*) is located approximately in the middle of the protein and serves to divide the molecule into the amino- and carboxyl-terminal halves. *B*, structure of TOM1 and its deletion mutants used in GST pull-down assays. TOM1 is organized into three domains: the amino-terminal VHS domain containing a single NPF motif, the central coiled-coil GAT region, and the carboxyl-terminal domain. *C*, interaction of endofin with TOM1. *Panel i*, recombinant GST, GST-TOM1(2–152), GST-TOM1(153–299), and GST-TOM1(300–492) were immobilized on glutathione-Sepharose beads and incubated with lysates of 293T cells transfected to express Myc-tagged full-length endofin. The beads were washed, and the associated proteins were resolved by SDS-PAGE followed by Western analysis with anti-Myc antibody. *Panel ii*, similar to *panel i* except that the GST or GST fusion proteins were incubated with lysate containing Myc-tagged endofin deleted of its amino-terminal half (Myc-Endofin(815–1539)). *Panel iii*, similar to *panel i* except that the GST or GST fusion proteins were incubated with lysate containing Myc-tagged endofin encoding only its amino-terminal half (Myc-Endofin(1–732)). *Panel iv*, Western analysis of the above GST pull-down experiments with anti-GST antibody. The blot is representative of all three experiments (*Panels i–iii*). *D*, residues in the TOM1 carboxyl terminus critical for interaction with endofin. Myc-tagged endofin prepared as above was incubated with GST fusion proteins of progressively deleted TOM1 carboxyl-terminal sequences as identified by the residue numbers (and schematically illustrated in *B*). Associated Myc-Endofin was detected by Western blot analysis (*upper panel*) and the GST fusion proteins used in the same experiment were revealed by Coomassie staining of the gel (*lower panel*).

control, was the only protein that was pulled down by GST-TOM1(300–492) bound to glutathione beads. Neither Myc-tagged SARA nor Hrs was detected in the GST-TOM1(300–492) pull-downs (Fig. 3). Hence, the interaction of TOM1 C terminus with endofin is likely to be a specific interaction.

Endofin Interacts Specifically with TOM1 but Not TOM1-L1 or TOM1-L2—A search of the data base reveals that TOM1 is a member of a family of three closely related proteins. The sequences of the two other members, namely TOM1-like 1 (TOM1-L1) (37, 39) and TOM1-like 2 (TOM1-L2) (accession number AF467441), have been reported. Alignment of the three sequences suggests a similar structural organization for the proteins, including a putative amino-terminal VHS and a central GAT domain (Fig. 4*A*). Closer examination of the protein sequences reveals that TOM1 is more similar to TOM1-L2 than TOM1-L1 (Fig. 4*B*). TOM1 is 76, 59, and 52% identical in amino acid sequence to TOM1-L2 in the VHS, GAT, and carboxyl-terminal region, respectively. In contrast, TOM1 is 43, 46, and 31% identical in sequence to the corresponding regions in TOM1-L1. It is evident that the region of greatest divergence between the three proteins is in the carboxyl-terminal region. This suggests that the TOM1 proteins may display unique binding specificities in their carboxyl termini.

In view of the similarities among the three TOM1 proteins, it is important to determine whether the association of endofin with TOM1 is unique to TOM1, while other members are excluded. We have cloned both the TOM1-L1 and TOM1-L2 cDNAs from human fetal brain by PCR. For comparative purposes, the TOM1-L1 and TOM1-L2 carboxyl-terminal sequences (amino acids 285–476 and 271–475, respectively) that are analogous to the carboxyl-terminal region in TOM1 that shows interaction with endofin were expressed as fusion proteins with GST. The GST-conjugated TOM1, TOM1-L1, and TOM1-L2 carboxyl-terminal proteins were then used in pull-down experiments with whole cell lysates from 293T cells transfected with Myc-tagged full-length endofin as described before. Only GST-TOM1(300–492) showed binding to Myc-tagged endofin, whereas GST alone or GST fused to TOM1-L1(285–476) or TOM1-L2(271–475) failed to do so (Fig. 4*C*). These data confirm the prediction that the carboxyl-terminal regions of the TOM1 proteins encode for unique binding specificities and hence confer functional differences for the three proteins.

Endofin Co-immunoprecipitates with TOM1—To facilitate an in-depth study of the relationship between endofin and TOM1, we generated polyclonal antibodies to the carboxyl-terminal 192 amino acids of TOM1 (Fig. 5*A*). Antibodies to this region of TOM1 are predicted to be specific for this protein because it is not similar to any other proteins in the data base at the time the antigen construct was designed, and it is obviously divergent from TOM1-L1. The antibody was purified by affinity chromatography and tested for specificity by Western analysis. Whole cell lysates from 293T cells transfected to express FLAG-tagged full-length TOM1 were subjected to SDS-PAGE followed by Western blotting with the purified anti-TOM1 antibody. The antibody was preincubated with an irrelevant TOM1 fragment fused to GST (GST-TOM1(2–152)), with the antigen (GST-TOM1(300–492)), or with GST-conjugated carboxyl terminus of TOM1-L1 (GST-TOM1-L1(285–467)) before adding to the blot to test for specificity (Fig. 5*B*, *left panel*). In lysates from untransfected cells, the antibody that was preincubated with the irrelevant proteins recognized a single band of about 50 kDa, corresponding to the predicted size of endogenous TOM1. A protein band of slightly slower electrophoretic mobility was detected in lysates from FLAG-tagged TOM1-transfected cells. This latter protein band represents

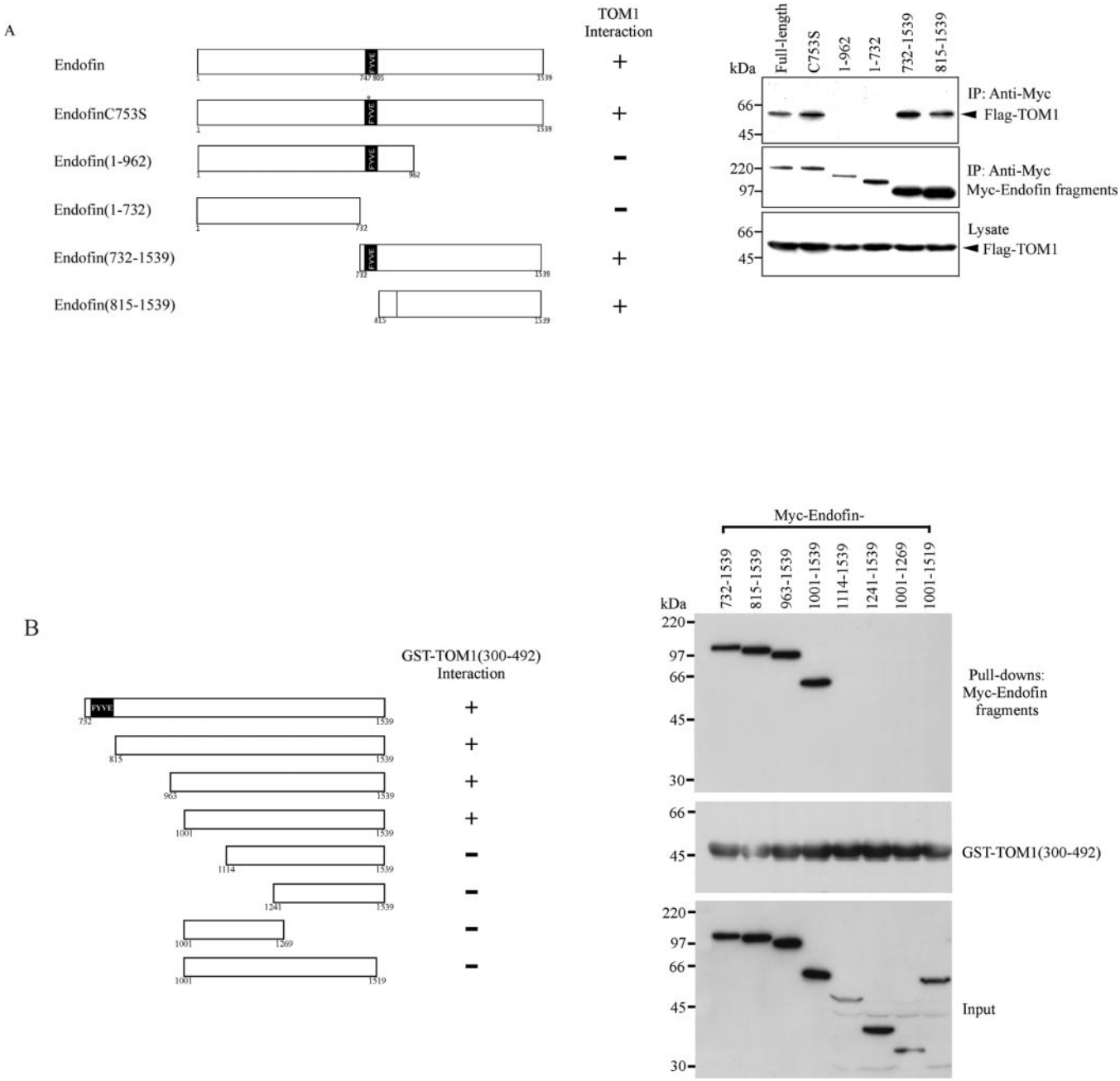

FIG. 2. **Interaction of endofin with TOM1 involves at least two distinct regions in its carboxyl-terminal half.** *A*, schematic representation of endofin and its deletion mutants used in the co-immunoprecipitation experiments with TOM1 and the importance of the carboxyl-terminal half of endofin in this interaction. 293T cells were co-transfected with plasmids encoding FLAG-tagged TOM1 and Myc-tagged full-length endofin or its truncated fragments shown in the *left panel*. Lysates of the transfected cells were prepared and subjected to immunoprecipitation with anti-Myc antibody and protein A-Sepharose beads. The beads were washed, and the associated proteins were resolved by SDS-PAGE followed by Western analysis with anti-FLAG (*right panel*, *top lane*) or anti-Myc (*right panel*, *middle lane*) antibody. An aliquot of each of the lysates used was also analyzed by Western analysis with anti-FLAG antibody (*right panel*, *bottom lane*). *B*, schematic representation of more endofin carboxyl-terminal truncated fragments used in the pull-down analysis and the requirement of two separate sequences in the endofin carboxyl-terminal domain. 293T cells were transfected with the Myc-tagged endofin deletion fragments shown in the *left panel* and the lysate prepared from each transfection was incubated with GST-TOM1(300–492) immobilized on beads. The associated Myc-tagged fragments as well as the inputs were detected by Western blotting (*right panel*) with anti-Myc antibody, whereas the GST fusion proteins were visualized by blotting with anti-GST antibody.

FLAG-tagged TOM1 as it was detected by the anti-FLAG antibody. The smaller protein species detected in the lysates of these transfected cells are likely to be proteolytic degradation products because they are also recognized by the anti-FLAG antibody (Fig. 5*B*, *right panel*). In contrast, when the antibody was preincubated with the antigen, endogenous as well as tagged TOM1 were no longer detected. These data establish that the anti-TOM1 antibody is able to specifically recognize the endogenous TOM1.

We also examined the ability of the TOM1 antibody to specifically recognize itself to the exclusion of the other family members. Potential cross-reactions to the other family members were tested by overexpressing the HA-tagged forms of all three TOM1 proteins followed by Western-blotting the SDS-PAGE-resolved proteins with the anti-TOM1 antibody. In addition to the endogenous TOM1 protein specie, the anti-TOM1 antibody was found to detect only the electrophoretically slower overexpressed HA-TOM1 protein but not HA-TOM1-L1

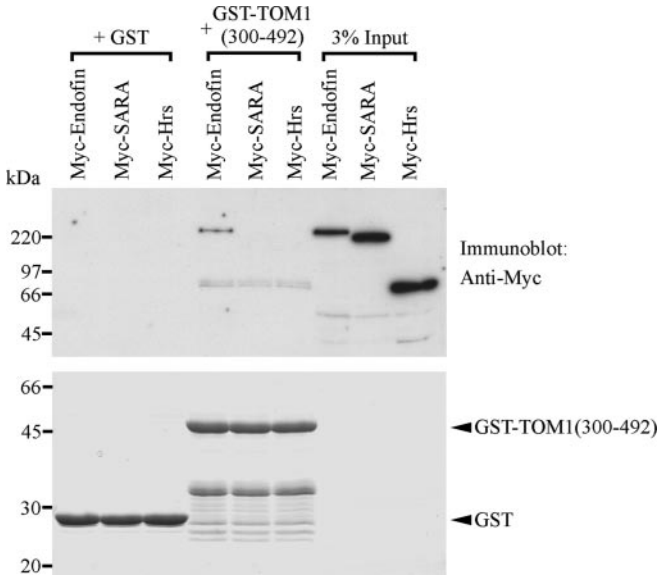

FIG. 3. **Neither SARA nor Hrs interacts with the carboxyl region of TOM1.** Myc-tagged endofin, SARA, or Hrs were overexpressed in 293T cells, and the individual lysates were incubated with either GST or GST-TOM1(300–492) immobilized on beads. The proteins bound to the beads were resolved by SDS-PAGE, and the associated Myc-tagged proteins were visualized by Western blotting with anti-Myc antibody (*top panel*). The GST or GST-TOM1(300–492) fusion proteins used in the experiment were detected by Coomassie staining of the gel.

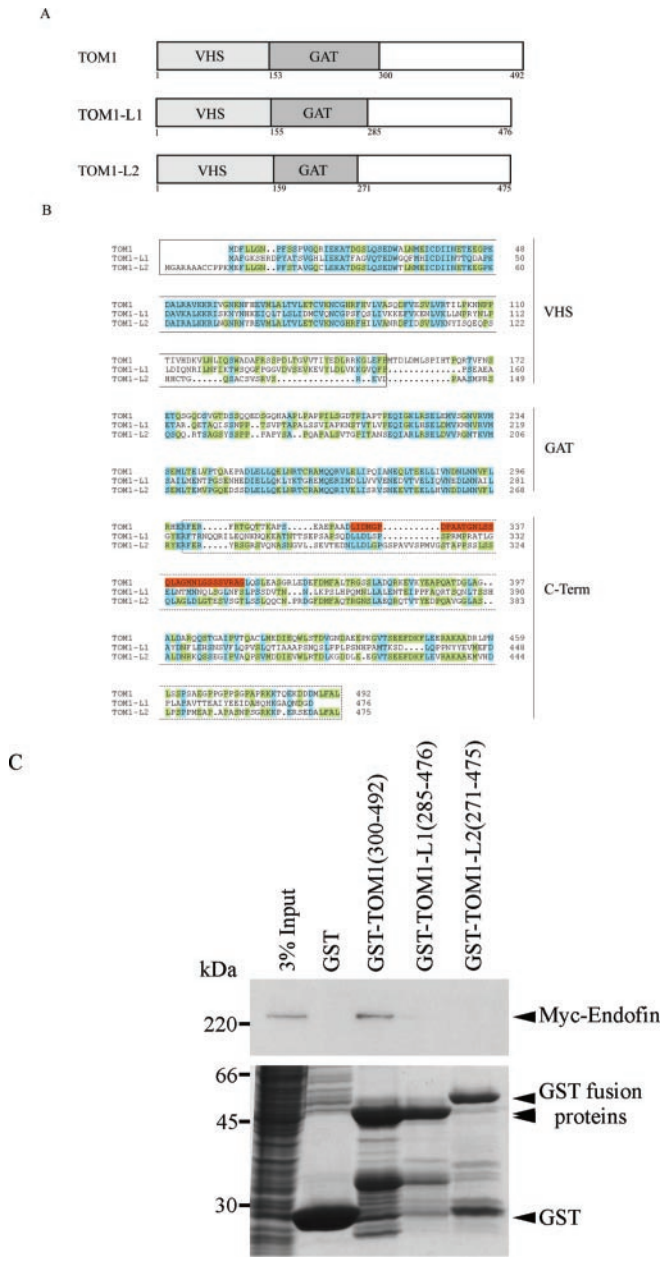

FIG. 4. **Endofin interacts specifically with TOM1 but not TOM1-L1 or TOM1-L2.** *A*, schematic structures of TOM1, TOM1-L1, and TOM1-L2 demonstrate a similarity in organization of the three major domains. *B*, alignment of the amino acid sequences of the TOM1 family members. The VHS domain is blocked in a *solid rectangle*, whereas the carboxyl-terminal region is blocked in a *dotted rectangle*. The residues in TOM1 that are critical for interaction with endofin are highlighted in *red*; residues conserved among all three members are highlighted in *blue*; residues that are conserved between any two members are highlighted in *green*. *C*, GST pull-down assays using TOM1, TOM1-L1, or TOM1-L2 carboxyl-terminal regions. Recombinant GST, GST-TOM1(300–492), GST-TOM1-L1(285–476) or GST-TOM1-L2(271–475) were immobilized on glutathione-Sepharose beads and incubated with lysates of 293T cells transfected to express Myc-tagged full-length endofin. The beads were washed, and the associated proteins were resolved by SDS-PAGE followed by Western analysis with anti-Myc antibody (*top panel*). The GST or GST fusion proteins used in the same experiment were visualized by Coomassie staining (*bottom panel*).

or HA-TOM1-L2 (Fig. 5*C*). The anti-TOM1 antibody is thus specific to and recognizes only TOM1.

The availability of the TOM1-specific antibody has allowed us to determine whether endogenous endofin is associated with the native TOM1. To do this, we performed co-immunoprecipitation experiments using whole cell lysates from A431 cells that were incubated with either anti-TOM1 or anti-endofin antibody (3) as control. The proteins bound to the antibodies were resolved by SDS-PAGE followed by Western blotting with anti-endofin antibody (Fig. 5*D*). The anti-TOM1 antibody was clearly able to co-immunoprecipitate endogenous endofin, demonstrating that endogenous endofin and TOM1 are associated with each other in the cell. Similar immunoprecipitation experiments using control rabbit IgG did not yield any signal for endofin (data not shown). We have also attempted to detect TOM1 in anti-endofin immunoprecipitates, but the signal was either very weak or hard to detect (results not shown). It is possible that the anti-endofin antibody may interfere with the binding of TOM1 to endofin.

TOM1 Is a Predominantly Cytosolic Protein—The anti-TOM1 antibody also allowed us to determine the intracellular localization of TOM1. We first performed indirect immunofluorescence analyses of a number of commonly used laboratory cell lines: 293T, A431, HeLa, BHK21, Chinese hamster ovary, and NRK cells (Fig. 6*A*). TOM1 is mainly distributed in the cytoplasmic region in all the cell types. Certain internal structures may be observed, but they are in general too indistinct to be identifiable.

We then proceeded to determine the intracellular localization of TOM1 by density gradient centrifugation. By simultaneously identifying the distributions of endofin and EEA1 as markers for early endosomes on a discontinuous 10–40% sucrose density gradient, we can elucidate whether endofin and TOM1 are located in similar intracellular regions for the relevant interactions to occur (Fig. 6*B*). Endofin, as expected, has a distribution pattern similar to that of EEA1. They therefore mark the fractions containing components of the early endosomal compartments. On the other hand, TOM1 distribution only partially overlaps with that of endofin, and its expression profile is shifted toward fractions of lower densities (see graphical presentation in the *lower panel*). These results indicate that TOM1 may reside partially on membrane structures with densities similar to that of the early endosomes where endofin is located.

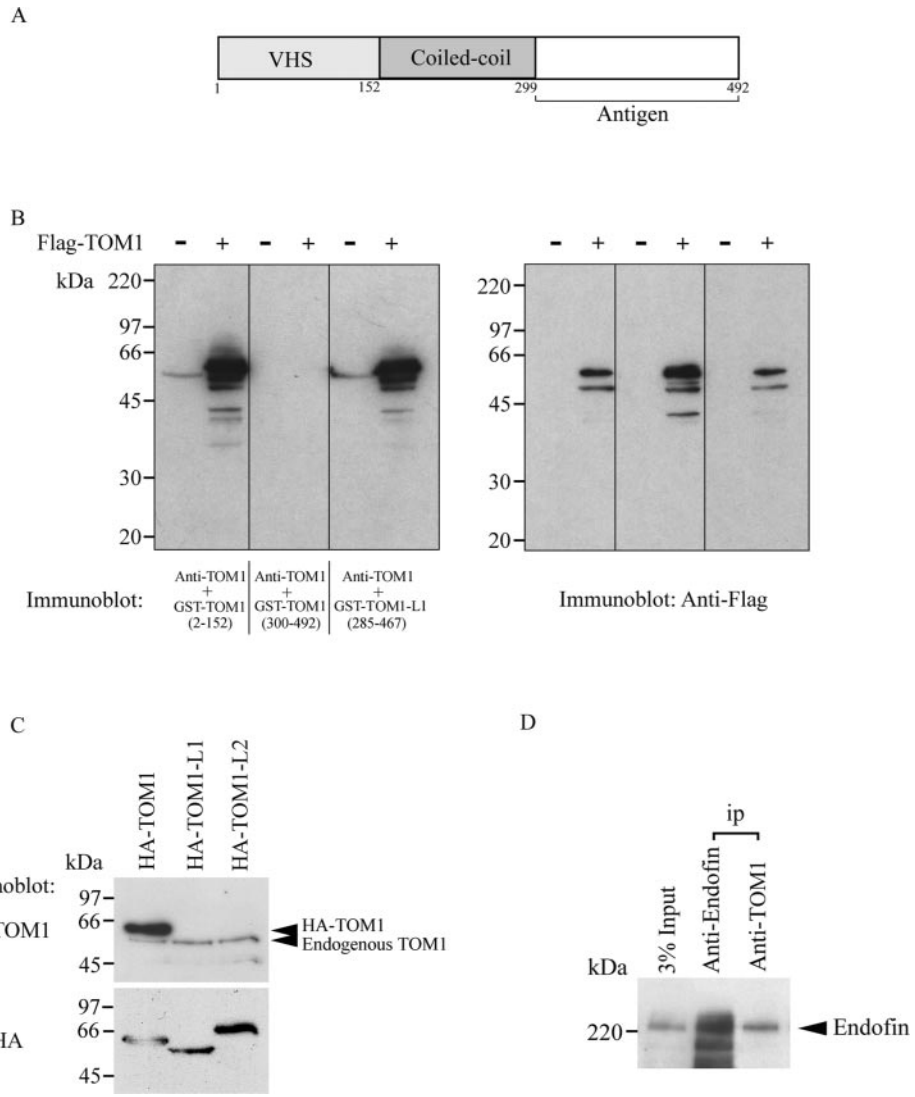

FIG. 5. **Antibody specific for TOM1 can immunoprecipitate endogenous endofin.** *A*, schematic structure of TOM1. The carboxyl-terminal region used as antigen to immunize rabbits for generation of TOM1-specific antibodies is indicated. *B*, Western analysis of wild-type 293T cells (−) or cells that were transfected with the plasmid encoding FLAG-tagged TOM1 (+) with anti-TOM1 antibody. The antibody was preincubated with either GST-TOM1(2–152) as a negative control, with the immunogen GST-TOM1(300–492) or with GST-TOM1-L1(285–476), prior to probing the blot (*left panel*). The TOM1 antibody recognizes a protein species of about 50 kDa in 293T cells and the slightly larger exogenously expressed FLAG-tagged TOM1. The same blot was probed with anti-FLAG antibody to confirm the electrophoretic profile of FLAG-tagged TOM1 (*right panel*). *C*, anti-TOM1 antibody can distinguish TOM1 from the other family members. Lysates from HA-tagged TOM1-, TOM1-L1-, or TOM1-L2-transfected 293T cells were resolved by SDS-PAGE and Western blotted first with anti-TOM1 followed by anti-HA antibody. The positions of the overexpressed HA-tagged TOM1 and of the electrophoretically faster endogenous protein are indicated by the *arrows*. *D*, co-immunoprecipitation of endogenous endofin and TOM1. A431 lysates were subjected to immunoprecipitation (*ip*) with either anti-endofin or anti-TOM1 antibody in the presence of protein A-Sepharose beads. The beads were washed, and bound proteins were eluted, resolved by SDS-PAGE, and Western blotted. The blot was probed with anti-endofin antibody.

Endofin Recruits TOM1 to Endosomes—To determine the possibility that endofin is able to recruit TOM1 to early endosomes *in vivo*, we expressed Myc-tagged endofin constructs in A431 cells and examined their effects on TOM1 localization by indirect immunofluorescence. As described previously (3), the overexpression of endofin causes aggregation of early endosomal structures (Fig. 7*A*). Interestingly, TOM1 is clearly recruited to these endosomal structures as opposed to nontransfected cells, which display the normal cytosolic appearance of TOM1. As expected, the cytosolic endofin C753S point mutant has no effect on TOM1 localization (Fig. 7*B*), whereas the carboxyl-terminal half of endofin (amino acids 732–1539), which contains the TOM1-binding site, is able to cause TOM1 recruitment (Fig. 7*C*). Overexpression of a short endofin fragment encoding mainly the FYVE domain sequence (amino acids 732–855; Fig. 7*D* and Ref. 3) has no significant effect on TOM1 localization, indicating that the recruitment of TOM1 to endosomal structures is dependent on and determined by its interaction with endofin. These results not only suggest that the interaction between endofin and TOM1 is physiologically relevant but also that one of cellular functions of endofin is to regulate endosomal targeting of TOM1.

DISCUSSION

Endofin is characterized mainly by the presence of a FYVE domain and a sequence similar to the Smad-binding domain present in SARA (30). This latter domain, however, did not seem to function in endofin as a Smad2-binding domain (3). Apart from these two motifs, there are no immediately obvious binding sites or motifs for known proteins. Indeed, a Blast search of both the amino- and carboxyl-terminal sequences of endofin for conserved motifs have revealed no results. Hence,

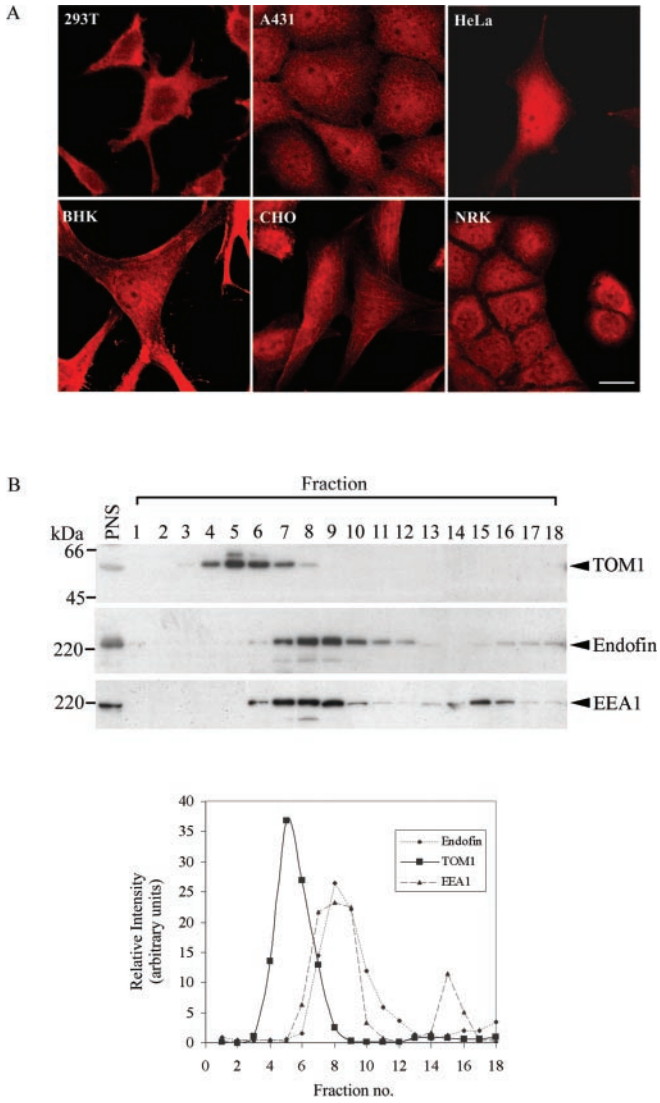

FIG. 6. **Analysis of TOM1 localization by both indirect immunofluorescence and isopycnic centrifugation on a discontinuous density gradient.** *A*, 293T, A431, HeLa, BHK21, Chinese hamster ovary (*CHO*), and NRK cells were fixed, permeabilized, and stained with anti-TOM1 antibody followed by visualization with Cy3-labeled anti-rabbit IgG. *Bar*, 10 μm. *B*, A431 cells were harvested to prepare a post-nuclear supernatant that was loaded on top of a 10–40% discontinuous sucrose gradient (see "Experimental Procedures"). After high speed centrifugation, the fractions were collected from the bottom of the tube. 25 μl of each fraction was then resolved by SDS-PAGE and Western blotted with anti-TOM1, anti-endofin, and anti-EEA1 antibodies. An aliquot of the post-nuclear supernatant (*PNS*) was also loaded as control. The fractions are numbered according to increasing densities (*top panel*). Band intensity was quantified by densitometry and expressed graphically (*bottom panel*).

we have embarked on a yeast two-hybrid screen for potential binding partners. In this study, we report the molecular characterization and cellular consequence of an interaction between endofin and TOM1.

The TOM1 proteins are hitherto little known proteins most closely related structurally to a well known family of proteins called the GGAs (40). Three GGAs (named GGA1, GGA2, and GGA3) have been identified in humans, whereas yeast is known to have two (Gga1p and Gga2p) and *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* have one each. The GGAs have a common structural organization of domains. They are composed of four modules: the amino-terminal VHS (VPS-27, Hrs, and STAM) domain, the GAT (GGAs and TOM1) homology domain, a flexible hinge region, and the carboxyl-terminal GAE (gamma-adaptin ear) domain. The VHS domain at the amino terminus is involved in interacting with specific acidic cluster/dileucine signals present in the cytoplasmic tails of cargo receptors. The GAT domain is responsible for binding to the GTP-bound form of ARF1 and ARF3 so that the targeting of the GGAs to the trans-Golgi network is regulated. The flexible hinge region contains clathrin-binding motifs. The carboxyl-terminal GAE domain is known to bind accessory proteins such as Rabaptin-5 (41) and γ-synergin (42). The GGAs are thus named because of their identification as Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding proteins. Although all of these domains are well characterized in the GGA proteins, little is known about the corresponding domains in the TOM1 proteins. For instance, the VHS domain of TOM1 does not recognize the acidic cluster/dileucine containing signal sequences and neither does its GAT domain bind ARF-GTP (43, 44).

The GGAs are mainly peripherally associated with the cytosolic portion of the *trans*-Golgi network as well as other vesicular structures unique to the individual GGAs (42, 45–48). It has been well demonstrated that it is at the level of the *trans*-Golgi network that the GGAs recognize and interact with specific cargos via their VHS domains and thereby mediate their sorting to vesicles destined to the endosomal/lysosomal pathway (43, 49–51). Our findings that TOM1 localizes mainly to the cytosol and the inability of its putative VHS and GAT domains to mimic the binding properties of those of the GGAs thus seem rather at odds with the general behavior of the GGA family of proteins, indicating that TOM1 have functions that deviate from the GGA proteins. Thus, although the function of the GGAs as adaptors for sorting receptors from the trans-Golgi network into the endosomal/lysosomal compartments is well characterized, the cellular distribution and functional role of TOM1 remain elusive.

Our finding that endofin recruits TOM1 to the endosomal compartment is therefore an important first step in the understanding of molecular mechanism of the function of endofin as well as the cellular role of TOM1. We have previously shown that endofin, when expressed at high levels, can cause endosomes to aggregate or fuse, and these structures can accumulate endocytosed epidermal growth factor and delay its degradation (3). In the present study, we demonstrate that these endosomal structures contain recruited TOM1. The effect on TOM1 is specific to endofin because similarly clustered or aggregated endosomal structures caused by overexpression of just the FYVE domain region of endofin lacking the TOM1-binding sequences do not recruit TOM1. Similarly, overexpression of SARA or Hrs fails to recruit TOM1 onto the aggregated endosomes (not shown). These data thus favor TOM1 as a new player in the endosomal trafficking pathway, the endosomal association of which is regulated by endofin.

By identifying TOM1 as a molecule that is recruited by endofin, we have also established one important role for endofin: it functions to regulate the subcellular localization and hence the function of TOM1. This regulatory function has also been attributed to SARA, a FYVE domain protein closely related to endofin. SARA has been well demonstrated to be important for recruiting Smad2 to the same endosomal compartment as the transforming growth factor receptor β receptor molecule, which then phosphorylates it and propagates the signaling cascade (30). Endofin may thus also function in an analogous fashion where it is critical for bringing together TOM1 and possibly its associated molecule(s) onto the endosomal membranes to regulate endocytotic trafficking or other pathways. The presence of the VHS and GAT domains, in conjunction with our observation that it interacts with endofin

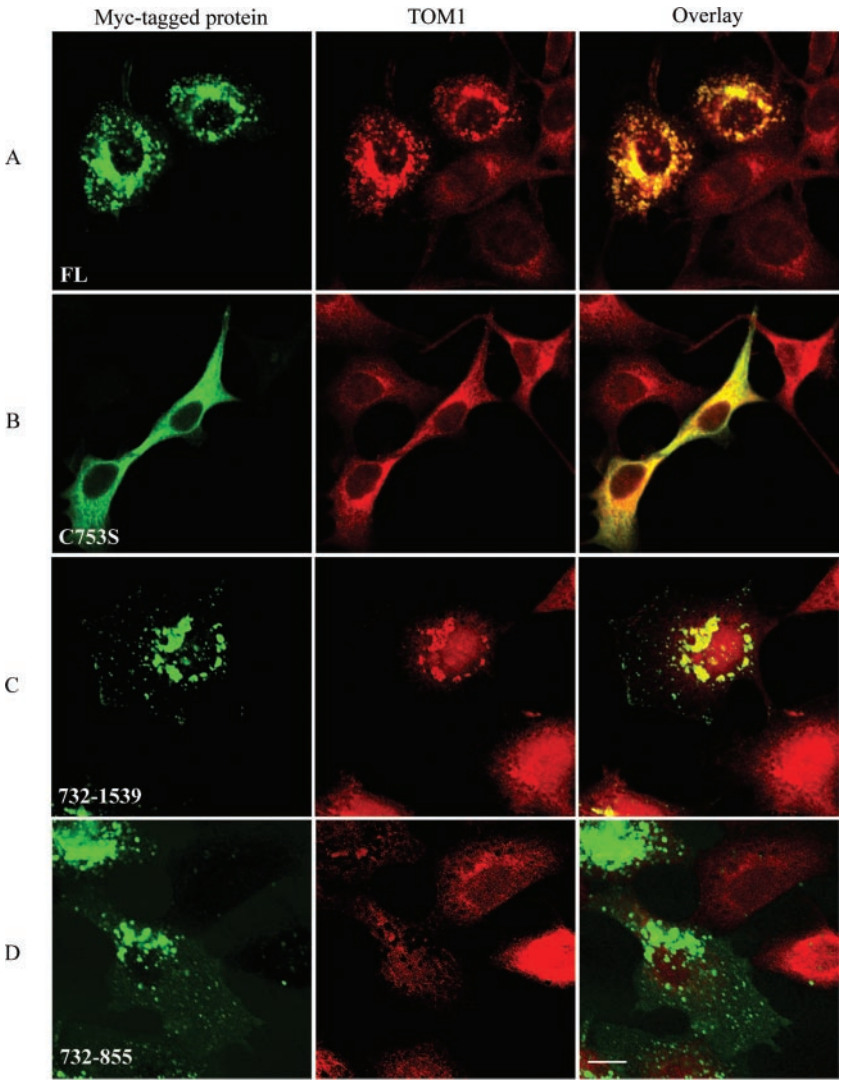

FIG. 7. **Overexpressed endofin recruits TOM1 to endosomes.** A431 cells were transfected with plasmids encoding Myc-tagged full-length (*FL*) endofin (*A*), endofinC753S (*B*), endofin(732–1539) (*C*), or endofin(732–855) (*D*). The cells were fixed, permeabilized, and stained with mouse anti-Myc (*left panels*) and anti-TOM1 (*middle panels*) antibodies followed by visualization with fluorescein isothiocyanate-labeled anti-mouse IgG and Cy3-labeled anti-rabbit IgG, respectively. Each *row* represents the same field, and merged images are shown in the *right panels* as overlays with *yellow* indicating co-localization. *Bar*, 10 μm.

via its unique carboxyl-terminal region, supports the view that TOM1 is likely to be associated with other proteins. It is also possible that endofin can serve to functionally define subendosomal compartments by recruiting TOM1 together with its associated proteins to specific areas of the endosomal membranes, a role that is also postulated for EEA1 because it forms unique complexes with different molecules. It follows that elucidating the identities of the proteins associated with TOM1 is crucial to defining the role of endofin in endosomal trafficking or any other pathways that it may potentially be involved in.

In this study, we have successfully identified a role for the carboxyl-terminal arm of endofin in recruiting TOM1 to endosomes. We have also defined the specific region of the carboxyl-terminal half of endofin as well as the residues in TOM1 that are involved in this interaction. The data gleaned from this study provide us with the basis for addressing other issues such as the nature of the cellular trigger that is required to effect a significant redistribution of TOM1 from the cytosol to the endosomal compartment under normal cellular settings. In addition to determining the spatial and temporal localization of TOM1 in relation to endofin and other cellular triggers, a resolution of the relationships between the endofin and other FYVE domain protein complexes on the endosomal compartment would be invaluable to our understanding of the process of endosomal trafficking.

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Membrane Transport, Structure, Function, and Biogenesis:

Endofin Recruits TOM1 to Endosomes

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