Endofin recruits clathrin to early endosomes via TOM1

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Summary

TOM1 and its related proteins, TOM1-like1 (TOM1-L1) and TOM1-like2 (TOM1-L2), constitute a subfamily of the VHS domain protein family. We have recently shown that endofin, a FYVE domain protein associated with the early endosome, is able to recruit cytosolic TOM1 onto endosomal membranes. To reveal the biological consequence of endofin-mediated endosomal recruitment of TOM1, we have identified the clathrin heavy chain as a major interacting protein for TOM1. Optimal clathrin binding by TOM1 involves three sites: residues 300-321, 321-326 and a putative clathrin-binding box at residues 362-366 (362LEDEF366). Although residues 321-326 could function independently as a weak clathrin-binding motif, deletion of amino acids 300-321 or mutation of 362Leu and 364Asp to Ala residues reduced the binding of clathrin to TOM1. A fragment lacking amino acids 300-322 and containing 362Leu and 364Asp to Ala mutations lost the ability to interact with clathrin. Remarkably, overexpression of endofin led to a massive and specific recruitment of clathrin [but not dynamin, or the adaptor protein (AP) complexes, AP1, AP2 or AP3] onto endofin-positive endosomes. Although SARA is homologous to endofin, it did not interact with the C-terminal region of TOM1. Examination of chimeric proteins of endofin and SARA suggests that the C-terminal half of endofin is responsible for interaction with the C-terminal region of TOM1 and for recruitment of TOM1 and clathrin to endosomes. The correlation between the ability of endofin to interact with the C-terminal domain of TOM1 and clathrin recruitment suggests that endofin may recruit clathrin via TOM1. Indeed, a chimeric protein consisting of TOM1 fused to two FYVE domains derived from endofin has the ability to recruit clathrin onto endosomal structures. Moreover, we show that affinity-purified TOM1 antibody can abolish binding of clathrin to the C-terminal region of TOM1. Upon microinjection into cells, this antibody reduced the membrane association of clathrin. These results, taken together, suggest that TOM1 is an important molecule for membrane recruitment of clathrin, and that endofin is able to exploit this recruitment at the endosome.

Key words: Endofin, TOM1, Clathrin, Endosomes, FYVE domain, VHS domain

Introduction

An emerging theme in cell biology is that many cytosolic proteins are dynamically recruited to the cytoplasmic side of specific membranes to mediate diverse intracellular processes. One major regulating mechanism is via the generation of various species of phosphoinositides (PIs) on the plasma membrane and the endosomes, leading to membrane recruitment of proteins that harbor domain(s) capable of interacting with the various PIs (Lemmon, 2003). For example, diverse signaling pathways initiated from ligand binding to the surface receptor results in the generation of phosphatidylinositol 3,4,5-trisphosphate by phosphoinositide 3-kinase on the plasma membrane, which in turn leads to the recruitment and activation of various protein kinases containing a PH domain such as PDK1 and AKT. Phox (PX) domain, FYVE domain, ENTH domain and several other structural domains or motifs are also conserved structures present in many different proteins that mediate membrane recruitment (Xu et al., 2001).

Endofin is a protein characterized by the presence of a phosphatidylinositol 3-phosphate (PI3P)-binding FYVE domain (Seet and Hong, 2001). It is recruited predominantly onto the early endosome and seems to regulate trafficking in the endocytic pathway, although the precise mechanism remains elusive. Major progress in defining the role and mechanism of the action of endofin in the endocytic pathway was made with the recent finding that endofin interacts with TOM1 (Seet et al., 2004). As this interaction leads to the recruitment of cytosolic TOM1 to the endosome, one of the roles of endofin is thought to involve the regulation of dynamic TOM1 recruitment onto the endosomes.

TOM1 is a member of a protein family characterized by the presence of the VHS (Vps27/Hrs/STAM) domain at the N-terminus (Lohi et al., 2002). TOM1 was originally identified as a target of Myb1 as the expression of TOM1 is dramatically induced upon expression of oncogenic Myb1 (Burk et al., 1997). Other members of the VHS protein family are TOM1-L1, TOM1-L2, Hrs, GGA1, GGA2, GGA3, STAM1 and STAM2. In addition to the VHS domain, TOM1, TOM1-L1, TOM1-L2, GGA1, GGA2 and GGA3 also contain a conserved GAT (GGA and TOM1) domain downstream of the VHS domain. GGA1, GGA2 and GGA3 are homologous proteins associated with the trans-Golgi network and other vesicular structures and they serve as effectors of ARF1 for recruitment onto the trans-Golgi network (TGN) (Bonifacino, 2004). The VHS domain of TGN-recruited GGA3 is known to interact...
with an acidic cluster-dileucine sorting signal present in the
cytoplasmic tail of mannos-6-phosphate receptor. This
interaction mediates sorting of the receptor into the endosomal
pathway so that lysosomal enzymes are specifically delivered
to the lysosomes via the endosomes (Puettolano et al., 2001; Nielsend et al., 2001; Zhu et al., 2001; Takatsu et al., 2001). On
the other hand, the VHS domain of TOM1 and its related
proteins do not appear to specify such a function nor do the
proteins serve as ARF effectors. The other VHS domain
proteins such as Hrs and STAM proteins interact with each other
and are shown to be enriched in the endosomes and
thought to function in integrating signaling pathway with
docytic traffic (Asao et al., 1997; Bache et al., 2003a).

In marked contrast to the GGAs, Hrs and STAMs, TOM1
and the related TOM1-L1 and TOM1-L2 are primarily
distributed to the cytosol (Seet et al., 2004; Yamakami et al.,
2003) (unpublished observations), suggesting that either
they function in the cytosol or their recruitment to specific
membranes is highly regulated. Interestingly, TOM1 was
recently reported to bind directly to ubiquitin, clathrin and
tollip, a mediator of interleukin-1 signaling (Yamakami et al.,
2003). However, as ubiquitination is associated with cargo
sorting on the endosomal-to-lysosomal membrane pathway
and clathrin functions as a membrane coat protein, the
implication of these interactions cannot be fully appreciated
against a cytosolic background.

Our recent demonstration that TOM1 is recruited onto the
endosome by its interaction with endofin (Seet et al., 2004)
suggests that other TOM1-interacting proteins may be
recruited to the endosomal membrane via endofin. In this
report, we have addressed the biological consequence of
endosomal recruitment of TOM1. We found that TOM1
interacts with clathrin and that it bridges endofin to clathrin
recruitment onto the endosome. The interactions involved are
highly specific. Our results show that optimal clathrin binding
by TOM1 involves three sites in its C-terminal domain.
Another point highlighted by our study is that the specific
interaction of TOM1 with endofin is critical for the endosomal
targeting of clathrin by endofin. Furthermore, microinjection
of TOM1 antibodies, which is able to specifically disrupt
interaction of TOM1 with clathrin, leads to an overall reduction
of membrane-associated clathrin.

Materials and Methods
Expression constructs

The construct containing glutathione S-transferase fused with the
C-terminus of TOM1 (amino acids 300-492) [GST-TOM1(300-492)] has
been described previously (Seet et al., 2004). All other GST fusion
constructs containing increasingly truncated TOM1 C-terminus
sequences or specific mutations were generated by PCR or by direct
ligation of oligonucleotides onto the pGEX-4T-1 vector (Amersham
Biosciences). The Myc-tagged endofin and SARA constructs were as
described (Seet and Hong, 2001). The Myc-tagged chimeric construct
of endofin-SARA contains the N-terminal 838 amino acids of endofin
fused to the C-terminal 633 amino acids of SARA whereas the
similarly tagged chimera of SARA-endofin consists of the N-terminal
693 amino acids of SARA ligated to the C-terminal 698 amino acids
of endofin. The HA-tagged chimeric construct of FYVE2-TOM1
consists of two FYVE domains of endofin (amino acids 747-805) fused
contiguously to the N-terminus of the full-length coding sequence
of TOM1. The HA-tagged chimeric constructs, FYVE2-
TOM1(L362,364A) where 362-Leu and 364-Asp of TOM1 were mutated
to Ala, FYVE2-TOM1Δ300-321 where amino acids 300-321 of TOM1
were deleted and, FYVE2-TOM1Δ300-321(L362,364A) where TOM1
was deleted of amino acids 300-321 as well as mutated with respect to
362-Leu and 364-Asp, were similarly generated by ligating two FYVE
domain coding sequences to the N-termini of the TOM1 cDNAs
containing the specified mutations. All constructs were verified by
dNA sequencing.

GST affinity chromatography

The affinity chromatography assay for identifying proteins binding to
the C-terminal region of TOM1 was adapted from a previously
described protocol (Li et al., 2002). Briefly, brains from 20 rats were
homogenized in BC buffer (25 mM Tris-HCl pH 7.8, 1 mM EDTA, 1
mM DTT) and the homogenates centrifuged at 200,000 g for 2 hours
in a SW41 Beckman rotor. The cytosol fraction was adjusted to 200
mM NaCl, 0.2% Igepal CA-630 and 1 mM PMSF and pre-cleared by
incubating with GST beads at 4°C, overnight. GST and GST-
TOM1(300-492) fusion proteins were produced in Escherichia coli
according to procedures described for the glutathione S-transferase
Gene Fusion System (Amersharm Pharamcia Biotech) and bound to
glutathione-Sepharose 4B beads (Amersham Pharmacica Biotech).
The GST (as negative control to identify any non-specific binding) or GST-
TOM1(300-492) affinity column containing 300 μg protein was pre-
washe extensively with BC500 (25 mM Tris-HCl pH 7.8, 500 mM
NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.2% Igepal CA-630, 1%
TX-100, 0.1% deoxycholate) followed by equilibration in BC200
(25 mM Tris-HCl pH 7.8, 200 mM NaCl, 1 mM EDTA, 1 mM DTT,
10% glycerol, 0.2% Igepal CA-630). Each column was then incubated
with 85 mg pre-cleared rat brain cytosol at 4°C, overnight. After
washing extensively with BC200, proteins bound to the columns
were eluted with BC500. The eluates were subjected to trichloroacetic
acid (TCA) precipitation and resolved by SDS-PAGE followed by
Coomassie Blue staining. Protein bands were cut out from the gel and
identified by mass spectrometry. Control experiments were performed
simultaneously by incubating the GST or GST-TOM1(300-492)
column with BC200 instead of rat brain cytosol.

Cell culture and transient transfection

293T and A431 cells were cultured as described previously (Seet and
Hong, 2001). Transfection of 293T cells was carried out using the
Lipofectamine2000 reagent (GibcoBRL) for about 5 hours and cells
were processed for immunofluorescence or western analyses 48 hours
after transfection.

Preparation of cytosol from cultured cells

Cells grown on 100-mm dishes were washed first with PBS and then
with cytosol buffer (0.2 M sucrose, 25 mM HEPES pH 7.0, 125 mM
potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, 0.2 mM
PMSF). The cell monolayer was then scraped into the residual buffer
remaining on the dish and homogenized by passing several times
through a 26- or 27-gauge needle. The broken cell suspension was then
centrifuged at 500 g for 5 minutes to obtain the post-nuclear supernatant
(PNS). The cytosol extract was eventually obtained by a subsequent
high-speed centrifugation of the PNS at 300,000 g for 1 hour in a Beckman SW60 rotor. The protein concentration of the
cytosol extract was measured using the Coomassie Plus Reagent
(Pierce).

Clathrin-binding assay

The clathrin-binding assay with GST fusion protein was adapted from
the protocol described in a previous report (Mullins and Bonifacino,
2001). Cytosol extract was incubated with GST or GST fusion protein
bound to beads for 4 hours at 4°C. After extensive washing in cytosol buffer, the beads were resuspended in 4x sample buffer and denatured at 95°C for 5 minutes. Samples representing 20 µg beads were then resolved by SDS-PAGE and analyzed by western blotting with anti-clathrin heavy chain antibody (Santa Cruz). An aliquot containing 50 µg of the original cytosol extract was also analyzed. To determine clathrin depletion, aliquots containing 50 µg protein were recovered from the post-pull-down supernatants, precipitated with TCA, and analyzed by SDS-PAGE and western blotting with anti-clathrin and anti-β-tubulin antibodies. To assay the effect of anti-TOM1 on clathrin binding by GST-TOM1(300-492), 20 µg GST-TOM1(300-492) bound to beads was pre-incubated with zero or the indicated amount of anti-TOM1 (1, 2, 5, or 10 µg) for 4 hours at 4°C. After washing three times with cytosol buffer, each sample was incubated with 500 µg A431 cytosol extract for 4 hours at 4°C. The beads were then washed again with cytosol buffer, resuspended in 4x sample buffer and 50 µg of each sample was analyzed by western blotting with anti-clathrin heavy chain and anti-rabbit conjugated to horseradish peroxidase to visualize the anti-TOM1 bound to the beads. 50 µg of the starting cytosol extract was also analyzed in the same experiment as before.

**Antibodies**

The polyclonal anti-clathrin heavy chain antibody for western blotting and monoclonal anti-clathrin heavy chain for immunofluorescence analysis were obtained from Santa Cruz and Research Diagnostics, respectively. Polyclonal anti-GST was from Amersham Pharmacia Biotech; monoclonal anti-β-tubulin (clone TUB 2.1) and anti-AP1 (clone 100/3) were purchased from Sigma-Aldrich. Anti-β-tubulin was as well as the anti-Myc tag used for immunofluorescence and western blot analyses were from Upstate Biotech. Anti-dynamin (Hudy 1) was also from Upstate Biotech and anti-AP2 and anti-AP3 were from Affinity Bioreagents and BD Transduction Laboratories, respectively. The rabbit anti-TOM1 antibody was raised as described previously (Seet et al., 2004). The AffiniPure rabbit anti-mouse IgG obtained from Jackson ImmunoResearch Laboratories was dialyzed extensively against PBS before being used for microinjecting cells. The secondary fluorescein isothiocyanate (FITC)-anti-rabbit and Cy3-conjugated anti-mouse antibodies were from Jackson ImmunoResearch Laboratories.

**Immunofluorescence analysis by confocal microscopy**

Cells were cultured and processed for immunofluorescence studies as described previously (Seet and Hong, 2001). In experiments where cells were stained with anti-clathrin, anti-Dynamin, anti-AP1, anti-AP2 or anti-AP3, the fixed cells were permeabilized with 0.05% saponin for 30 minutes. Otherwise, permeabilization was achieved by incubating the fixed cells in 0.1% Triton X-100 for 5 minutes at room temperature. Cells were viewed using a confocal laser-scanning microscope (Zeiss).

**Microinjection**

A431 cells were cultured overnight on coverslips. Just before microinjection, the culture medium was replaced with fresh medium containing additional 20 mM HEPES, pH 7. Anti-TOM1 or rabbit anti-mouse IgG (as control) were microinjected into the cells using a Transjector 5246 (Eppendorf, Germany) aided by a micromanipulator 5171 (Eppendorf, Germany) and a Zeiss Axiovert 35 microscope. The cells were allowed to recover for 2 hours in a 37°C humidified incubator before being processed for immunofluorescence studies.

**Results**

**TOM1 interacts with clathrin**

In order to understand the consequence of endofin-mediated endosomal targeting of TOM1, we have used the GST pull-down affinity chromatography method to identify proteins that interact with the C-terminal region of TOM1. The C-terminal fragment (residues 300-492) of TOM1 was expressed as a fusion protein with GST and immobilized on glutathione beads. Immobilized GST-TOM1(300-492) and GST (serving as a negative control) were separately incubated with rat brain cytosol to retrieve proteins that interact specifically with the C-terminal region of TOM1 (Fig. 1A). Strikingly, only one major protein species of about 220 kDa was specifically recovered by the GST-TOM1(300-492) beads. This was absent in both the GST or control beads. Other retained proteins that were present in both the GST-TOM1(300-492) as well as GST beads represent non-specific interactions. Mass spectrometric analysis of the 220 kDa protein species cut out from the Coomassie-stained gel identified it as the clathrin heavy chain.

To confirm the interaction and to evaluate the efficiency of retrieval of clathrin heavy chain by GST-TOM1(300-492), analytical GST pull-down experiments were performed and a representative result shown in Fig. 1B. Clathrin heavy chain was very efficiently retained by the GST-TOM1(300-492) beads (right lane, top panel) when compared to the amount of input material (4%, left lane, top panel) used in the experiment. This is also clearly reflected by the dramatic reduction of clathrin heavy chain left in the post-pull-down extract (right lane, middle panel) relative to that remaining after incubation with the control GST beads (central lane, middle panel). These results suggest that clathrin is a specific partner for TOM1.

**Optimal interaction of TOM1 with clathrin is mediated by the concerted action of three sites in the C-terminus of TOM1**

We next examined the structural basis of the interaction between TOM1 and clathrin. To do this, we created TOM1 deletion mutants that were progressively truncated with respect to the C-terminal region (Fig. 2A). These mutants were expressed as GST fusion proteins and their abilities to retain clathrin were assessed by analytic GST pull-down assays. As shown in Fig. 2B, deletion of residues 300-321 reduced significantly but did not abolish the retention of clathrin (lane 4) as compared to GST-TOM1(300-492) (lane 3). Further deletions up to residue 361 had no noticeable effect on this clathrin retention (lanes 5-7). However, additional deletion of residues 362-366 abolished essentially most of the detectable clathrin binding (lane 8). These results suggest that there is an independent clathrin-binding site around residues 362-366, whereas residues 300-321 either plays an enhancing role for this binding site or they contain an independent binding site for clathrin. To distinguish between these two possibilities, another series of GST fusion of TOM1 C-terminal mutants were analyzed. A scan of the residue sequence reveals that it is related to the conserved clathrin-binding box found in several proteins (Dell’Angelica et al., 1998). Hence, we proceeded to mutate the conserved Leu and Asp residues at positions 362 and 366 into Ala in GST-TOM1(300-492) and GST-TOM1(322-492) (Fig. 3A). As shown in Fig. 3B, mutation of this putative clathrin box in the context of GST-TOM1(300-492) reduced but did not abolish clathrin binding (lane 4) when compared to GST-TOM1(300-492) without the mutations (lane 3). However, when this motif was mutated in
the context of GST-TOM1(322-492), clathrin binding was essentially abolished (lane 6) whereas the interaction was observable in the version without the mutations (lane 5). These results suggest that LEDEF is indeed a functional clathrin-binding motif in the region of residues 322-492. In addition, as mutation of the clathrin-binding box in GST-TOM1(300-492) did not abolish clathrin binding (lane 4) whereas the GST-TOM1(322-492)L362,364A mutant failed to interact with clathrin (lane 6) suggests that the region encompassing residues 300-321 can serve either as an independent clathrin-binding site or as an enhancer for clathrin binding.

A previous study has identified residues DLIDMG of TOM1 as a binding site for clathrin (Yamakami et al., 2003). To ascertain if residues 300-321, DLIDMG or LEDEF constitute independent clathrin-binding domains, we designed GST fusion constructs expressing the respective peptides (Fig. 4A). As observed in Fig. 4B, residues 300-321 (lane 5), 327-366 (lane 8) and 362-366 (lane 9) could not pull down clathrin. This indicates that residues 300-321 and the LEDEF motif could not function independently per se as clathrin-binding domains. It remains possible, however, that

the 362LEDEF motif may be able to function independently if additional C-terminal amino acids are present. On the other hand, the DLIDMG motif could function independently to interact with clathrin, albeit extremely weakly (lanes 6 and 15). This interaction is greatly enhanced when residues 300-321 were present (lanes 4 and 14) and moderately improved when residues 327-366 were included (lanes 7 and 16). Even better interaction was observed when both residues 300-321 and the C-terminal residues up to amino acid 366 were present (lanes 3 and 13). This level of interaction is almost comparable to that observed when the entire C-terminal domain of TOM1 was used (lane 12). In addition, comparison of the clathrin-binding efficiencies of residues 321-366 (lane 16) and 322-492 (lane 17) supports the possibility that LEDEF may function more efficiently in the presence of additional C-terminal residues whereas additional N-terminal residues as represented by 327-366 (lane 8) have no effect. Taken together, these results suggest that optimal clathrin binding by TOM1 involves three sites: residues 300-321, which seems to play an enhancing role; the DLIDMG motif, which resembles a canonical clathrin-binding box and possesses the ability to bind clathrin independent of other residues; and LEDEF, which...
which could not function independently per se but could augment clathrin association.

These data, together with our previous study that identified a region spanning residues 322-353 in TOM1 as important for interaction with endofin (Seet et al., 2004), provides the following domain organization of the TOM1 C-terminus: an endofin-binding domain (EBD) sandwiched between three sites crucial for clathrin binding (CB, CB-I and CB-II, Fig. 4C).

Endofin mediates specific recruitment of clathrin onto endosomes

As endofin can mediate endosomal recruitment of TOM1 to early endosomes (Seet et al., 2004), we examined whether overexpression of endofin has any effect on the distribution of clathrin. Immunofluorescence analysis of A431 cells overexpressing Myc-tagged endofin revealed that clathrin heavy chain is massively recruited onto endosomes marked by endofin (Fig. 5A). Interestingly, this effect is specific to clathrin as overexpression of endofin had no effect on the distribution of the coated vesicle-associated GTPase, dynamin (Fig. 5B) or the clathrin-associated adaptor proteins, namely AP1 (Fig. 5C), AP2 (Fig. 5D) or AP3 (Fig. 5E).

Interaction with TOM1 is necessary for endofin to recruit clathrin

The above data suggest that endofin may recruit clathrin via TOM1 as the adaptor. To explore this possibility, we designed our experiments based on the knowledge that SARA (Tsukazaki et al., 1998), a FYVE domain protein that is closely related to endofin, does not bind to the C-terminal region of TOM1 (Seet et al., 2004). In addition, as the FYVE domain in both proteins appears to divide them into two halves (the N- or C-terminal half), chimeric proteins consisting of either the N-terminal half of endofin ligated to the C-terminal half of SARA (endofin-SARA) or vice versa (SARA-endofin) provide useful tools for testing the differential functional properties of the proteins as contributed by either the N- or C-terminal segment of the molecules (Fig. 6A). With this rationale in mind, the abilities of overexpressed Myc-tagged endofin, SARA and the two chimeric proteins to interact with GST-TOM1(300-492) were compared and their effects on the endosomal recruitment of clathrin were correspondingly examined. As shown in Fig. 6B, only Myc-endofin and Myc-SARA-endofin, but not Myc-SARA or Myc-tagged endofin-SARA were retained by immobilized GST-TOM1(300-492). These data are consistent with our previous finding that the C-terminal region of endofin is responsible for interaction with TOM1 whereas SARA cannot interact with the C-terminal domain of TOM1 (Seet et al., 2004).

Endofin, SARA and both chimeras described above are associated with endosomal structures as they all contain the FYVE domain. To study the correlation between the ability to interact with TOM1(300-492) in GST pull-down assays and TOM1/clathrin recruitment in cells, we examined A431 cells overexpressing each of the above Myc-tagged wild-type or chimeric constructs by immunofluorescence analysis. In line with the pull-down results above, only the GST-TOM1(300-492)-interacting proteins, that is, Myc-endofin and Myc-SARA-endofin, were
able to recruit TOM1 onto the endosomes when overexpressed in A431 cells (Fig. 6C, i and iv). On the other hand, Myc-SARA and Myc-endofin-SARA, which were not pulled down by GST-TOM1(300-492), had no effect on the cytosolic distribution of TOM1 (Fig. 6D, ii and iii). These data suggest that the ability of endofin to recruit TOM1 to endosomes is necessary for clathrin membrane recruitment. It is therefore highly likely that endofin recruits clathrin via TOM1.

A chimera of TOM1 fused to two FYVE domain sequences is localized in early endosomes

To confirm that TOM1 is indeed able to recruit clathrin in vivo, we set up experiments based on an exogenously expressed form of TOM1 whose targeting to early endosomes is autonomous. This endosomal-associated TOM1 is generated by fusing TOM1 to two FYVE domain sequences at its N-terminus (Fig. 7A). The FYVE domain binds specifically to phosphatidylinositol 3-phosphate and it has been reported that expression of a construct containing a double FYVE finger of Hrs (instead of a single FYVE domain) binds with higher affinity to the phospholipid as well as labels intracellular endocytic vesicles (Gillooly et al., 2000). The FYVE domain sequence used in this report was derived from endofin that we have previously shown to be sufficient for the endosomal localization of endofin as well as to cause endosomal aggregation/fusion (Seet and Hong, 2001). When overexpressed in A431 cells and examined by immunofluorescence analysis, the HA-tagged TOM1 chimera (HA-FYVE2-TOM1) was able to cause aggregation/fusion of vesicular structures containing the early endosomal marker EEA1 (Fig. 7B) as well as endofin (Fig. 7C). Importantly, further analyses using markers for the clathrin-associated adaptor proteins AP-1 (Fig. 7D), AP-2 (Fig. 7E) and AP-3 (Fig. 7F) revealed that HA-FYVE2-TOM1 behaved like wild-type endofin in having no significant effects on the distribution of these proteins as described above (Fig. 5). Taken together, these observations indicate that HA-FYVE2-TOM1 has essentially adopted the specific localization attributes of an endosomal protein like endofin as a result of the addition of the FYVE domain sequences. Thus, for the purposes of the following experiments, HA-FYVE2-TOM1 may be taken to represent endosomally-recruited TOM1 whose localization is otherwise normally regulated by endofin (Seet et al., 2004).

HA-FYVE2-TOM1 can recruit clathrin to endosomal structures and both CB-I and CB-II are critical for this activity

The positive characterization of HA-FYVE2-TOM1 allowed us to proceed to the next step: determination of the effect of overexpressing HA-FYVE2-TOM1 on the distribution of clathrin. Indeed, overexpression of HA-FYVE2-TOM1 was able to recruit endogenous clathrin heavy chain to vesicular structures, as assessed by immunofluorescence analysis (Fig. 8B,
Apart from confirming the clathrin-recruiting ability of TOM1, this result also suggested that the chimeric construct may be a useful means for determining the importance or necessity of CB-I and/or CB-II (see Fig. 4C) for the membrane recruitment of clathrin. Hence, we generated HA-tagged FYVE domain chimeric constructs of TOM1 mutated at either or both CB-I and CB-II sites (Fig. 8A). Overexpression of the chimera mutated at only CB-II where residues 362 and 364 were converted to alanine [HA-FYVE2-TOM1(L362,364A); Fig. 8B, ii] or the chimera deleted of CB-I [HA-FYVE2-TOM1Δ300-321; Fig. 8A].

**Fig. 4.** Optimal interaction of TOM1 with clathrin involves three sites: residues 300-321, 321-326 and 362-366. (A) Schematic diagram of TOM1 C-terminal deletion constructs used in the clathrin pull-down experiments. The relative efficiency with which each construct pulled down clathrin as observed in B is indicated on the right as + or −. (B) The clathrin-binding domain of TOM1 includes one putative clathrin-binding box at 321-326, which is sufficient by itself whereas residues 300-321 and 362-366 enhance the clathrin-binding efficiency. The GST fusion constructs shown in A were used to pull down cytosolic clathrin and analyzed as described in Fig. 2B. The upper panels indicate the amounts of clathrin pulled down by 20 µg of GST or GST fusion proteins. Input represents 50 µg of the starting material. The bottom panels are Coomassie-stained versions of the same experiments. (C) Schematic diagram depicting the location of the sites in TOM1 that are critical for optimal clathrin interaction. CB, clathrin-binding site; CB-I and CB-II: clathrin-binding enhancing sites I and II, respectively; EBD, endofin-binding domain; GAT, GGAs and TOM1 homology; VHS, VPS-27, Hrs and STAM.
8B, iii] was observed to be only weakly to moderately effective in recruiting clathrin to vesicles. The double mutant where both CB-I and CB-II were mutated [HA-FYVE2-TOM1Δ300-321(L362,364A)] was found to be largely ineffective in causing an obvious accumulation of clathrin in vesicles containing the overexpressed proteins (Fig. 8B, iv). These observations thus confirmed two important points: first, endosomal TOM1 has the ability to recruit clathrin and therefore may serve as an adaptor for endofin and clathrin; second, both CB-I and CB-II are critical for the clathrin-recruitment property of TOM1.

Antibodies against TOM1 inhibit interaction with clathrin
We have previously raised antibodies targeted specifically against the C-terminal region (residues 300-492) of TOM1 (Seet et al., 2004). As the clathrin-binding sites of TOM1 are located within this region, these antibodies may interfere with the interaction between TOM1 and clathrin. To determine this possibility, we carried out an analytic GST pull-down experiment in which a fixed amount (20 µg) of immobilized GST-TOM1(300-492) was pre-incubated with increasing amounts of TOM1 antibodies. After washing extensively, the beads were incubated with a constant amount (500 µg) of cytosol; the amount of clathrin heavy chain retained by the beads was then analyzed by western blot analysis. As shown in Fig. 9A, inclusion of 5-10 µg of antibodies can effectively abolish the interaction of clathrin with TOM1, whereas a similar amount of control rabbit IgG had no effect. These results not only suggest that our TOM1 antibodies are recognizing epitopes that overlay with sites critical for clathrin binding but also further confirmed that the interaction between TOM1 and clathrin is specific.

Microinjected TOM1 antibodies reduced membrane association of clathrin
As the TOM1 antibodies can interfere with clathrin binding to TOM1 in vitro, we were interested in examining the cellular effect of blocking this interaction in the cell. We addressed this issue by microinjecting the TOM1 antibodies into A431 cells. We then examined the effect of blocking the TOM1-clathrin interaction on the cellular distribution of clathrin by immunofluorescence analysis. In non-microinjected cells, clathrin appears to be associated with diverse membrane structures (Fig. 9B, i). This labeling pattern was however, greatly perturbed when the cells were microinjected with TOM1 antibodies. In more than 95% of these microinjected cells, the amount of membrane-associated clathrin was dramatically reduced (Fig. 9B, i). This effect is especially striking when compared to control cells microinjected with rabbit IgG where clathrin remains typically associated with diverse membrane structures (Fig. 9B, ii). Furthermore, microinjection of the anti-TOM1

Fig. 5. Overexpressed endofin recruits clathrin to early endosomes. A431 cells were transfected with Myc-tagged endofin and processed for immunofluorescence analysis by fixing followed by permeabilization with 0.05% saponin. The cells were stained with anti-Myc (left panels and green in merged images) and anti-clathrin heavy chain (A), anti-dynamin (B), anti-AP1 (C), anti-AP2 (D) or anti-AP3 (E) (middle panels and red in merged images on right) and analyzed by confocal microscopy. Areas of yellow coloration in merged images indicate colocalization. Bar, 10 µm.
Fig. 6. The C-terminal region of endofin cannot be replaced by the corresponding region of SARA for interaction with the C-terminal domain of TOM1 and its membrane recruitment. (A) Schematic of wild-type endofin and SARA constructs as well as the chimeric constructs used in the pull-down experiments. (B) The C-terminal region of endofin but not SARA mediates binding to the TOM1 C-terminus. 293T cells were transfected with the Myc-tagged constructs depicted in A and the resultant lysates were incubated with either GST or GST-TOM1(300-492), which were bound to glutathione-Sepharose beads. The inputs (3%) as well as pull-down materials were resolved by SDS-PAGE and analyzed by western blotting with anti-Myc (upper panel) and anti-GST (lower panel) antibodies, the latter to indicate levels of GST or GST fusion proteins used in the experiment. (C) Membrane recruitment of TOM1 by endofin is dependent on the C-terminus of endofin, which cannot be replaced by the corresponding region of SARA. A431 cells were transfected with each of the Myc-tagged constructs depicted in A. Upon fixation and permeabilization, the cells were stained with antibodies against the Myc epitope (left panels) and endogenous TOM1 (right panels). (D) Membrane recruitment of clathrin by endofin is dependent on the C-terminus of endofin, which cannot be replaced by the corresponding region of SARA. A431 cells were transfected with the Myc-tagged constructs depicted in A. Upon fixation and permeabilization, the cells were stained with antibodies against the Myc epitope (left panels) and clathrin heavy chain (HC) (right panels). Bar, 10 µm.
antibodies did not affect the distribution of dynamin or AP2 (data not shown), suggesting that the effect of anti-TOM1 antibodies on clathrin is specific and that an interaction between TOM1 and clathrin is important for clathrin membrane recruitment in the cell.

Discussion

In this report we have addressed the biological consequence of endofin-mediated TOM1 recruitment onto endosomes. Our results collectively suggest that TOM1 serves as an adaptor for endofin to recruit clathrin heavy chain onto the endosomes. This conclusion is supported by several lines of evidence as described in our study.

First, via large-scale pull-down experiments using immobilized GST-TOM1(300-492), we have recovered clathrin heavy chain as the major and specific partner for TOM1. This conclusion was corroborated by analytical pull-down experiments showing that clathrin heavy chain was very efficiently retained by immobilized GST-TOM1(300-492), so much so that it was depleted from the cytosol. The specific interaction between TOM1 and clathrin heavy chain was further defined by our identification of three sites in the carboxyl-terminal region of TOM1, which seem to act together for efficient interaction with clathrin. Moreover, the specific blockage of interaction between TOM1 and clathrin by antibodies against TOM1 (raised against the C-terminal region) further confirmed the specificity of this interaction.

Second, we observed that overexpression of endofin causes a massive recruitment of clathrin heavy chain onto endofin-marked endosomes. As TOM1 is recruited from cytosol to the endosomes when endofin is overexpressed (Seet et al., 2004), it follows that clathrin recruitment to endosomes may occur as a consequence of TOM1 recruitment by endofin. The ability of endofin to recruit clathrin is specific, as neither dynamin nor any of the clathrin-associated adaptor proteins AP1, AP2 or AP3, were recruited by overexpressed endofin. This is further shown by our observation that SARA, which is homologous to endofin, failed to recruit clathrin heavy chain. This may be explained by the fact that SARA does not interact with the C-terminus of TOM1 (Seet et al., 2004).

Third, we demonstrated that the ability of endofin to interact with TOM1 is necessary for it to recruit clathrin heavy chain. As SARA does not interact with the C-terminal region of TOM1 (Seet et al., 2004), we have examined the properties of chimeric proteins composed of either the N-terminal half of endofin fused to the C-terminal half of SARA or vice versa. A chimeric protein consisting of the N-terminal segment of SARA joined to the C-terminal half of endofin is able to interact with the C-terminal region of TOM1, as well as to recruit both TOM1 and clathrin. In contrast, the chimeric protein consisting of the N-terminal region of endofin fused to the C-terminal half of SARA did not interact with the C-terminus of TOM1 and failed to recruit TOM1 or clathrin onto endosomes. Thus, we have provided evidence to support the correlation between the ability to interact with the C-terminus of TOM1, as specified by the C-terminal domain of endofin, and the recruitment of TOM1 as well as clathrin.

Fourth, we reported the ability of TOM1 to recruit clathrin to endosomes when it is deliberately localized in the endosomal compartment via the fusion of two FYVE domain
sequences to its coding sequence. This property is dependent on both CB-I and CB-II, two sites that enhance the binding of clathrin to TOM1. Immunofluorescence analyses with the chimeric constructs suggested that mutation of either CB-I or CB-II significantly reduces the clathrin-recruitment activity of TOM1 whereas deletion of both sites more or less abolished this property. These data corroborate to suggest that TOM1, as it is being recruited by endofilin to the early

![Diagram](image)

**Fig. 8.** HA-FYVE2-TOM1 chimera recruits clathrin to endosomes while mutation of CB-I and CB-II reduces this activity. (A) Schematic diagram of the chimeric constructs used in the experiment. Mutated residues are indicated in italics and deletions are indicated by a gap. (B) HA-FYVE2-TOM1 recruits clathrin to endosomes whereas the chimeric CB-I and CB-II single or double mutants are significantly less effective. A431 cells were transfected with each of the constructs depicted in A and processed for immunofluorescence analysis by fixing with paraformaldehyde followed by permeabilization with 0.05% saponin. The cells were stained with anti-HA (left panels and green in merged images) and anti-clathrin heavy chain (middle panels and red in merged images) and analyzed by confocal microscopy. Merged images are shown in the right panels where yellow represents areas of colocalization. CB, CB-I and CB-II, clathrin-binding sites; EBD, endofilin-binding domain; GAT, GGAs and TOM1 homology; VHS, VPS-27, Hrs and STAM. Bar, 10 µm.
endosomes, can potentially recruit clathrin directly to the same membranes.

Finally, we showed that the association of clathrin heavy chain with membrane structures is dramatically reduced in cells where interaction between TOM1 and clathrin heavy chain is disrupted by microinjection of anti-TOM1 antibodies. This result thus highlights the intimate in vivo relationship between TOM1 and clathrin. In addition, this observation suggests that there exists a threshold level of membrane-associated TOM1 in the cell, possibly maintained by endofin, which is required to maintain a steady-state level of clathrin in membranous vesicular structures.

Taken together, we conclude that endofin recruits the clathrin heavy chain onto endosomes via TOM1 as the bridging adaptor. As TOM1 is predominantly cytosolic (Seet et al., 2004), its recruitment onto endosomes by endofin could be one of the regulatory loops in the membrane targeting of the clathrin heavy chain. As anti-TOM1 antibodies not only reduced the association of clathrin heavy chain with endosomes but also reduced the general membrane association of clathrin heavy chain, it is conceivable that the TOM1-clathrin relationship could be exploited by other proteins at endosomes and/or other membrane sites. Identification of other proteins that are capable of recruiting TOM1 onto membrane structures will be important areas for future investigation. In this regard, it is interesting to note that Tollip is an interacting partner for TOM1 (Yamakami et al., 2003). Tollip is transiently recruited onto the plasma membrane during signaling mediated by binding of interleukin-1 (IL-1) to its receptor complex (Burns et al., 2000). As Tollip interacts with the receptor complex on the plasma membrane, it will be of interest to examine whether IL1-triggered Tollip interaction with the receptor complex could cause a transient recruitment of clathrin heavy chain onto the membrane surface.

The same report that described the TOM1-Tollip Interaction also mentioned the association of TOM1 with clathrin heavy chain (Yamakami et al., 2003). However, the consequence of this interaction was not investigated in that study, although our results suggest that this interaction is important for endofin to recruit clathrin onto endosomes. It was concluded in that report that the clathrin-binding site in TOM1 is located at residues 321DLIDMG326 (Yamakami et al., 2003). Our unbiased systematic analysis of deletion and point-mutated fragments of the TOM1 C-terminal region has enabled us to identify two additional sites located at residues 300-321 and 362LEDEF366 that are critical for optimal clathrin binding. Furthermore, we have demonstrated that the reported 321DLIDMG326 motif can function independently as a clathrin-binding motif whereas the residues 300-321 are likely to play a supporting/enhancing role. Alternatively, it is possible that 321DLIDMG326 is the bare minimum for clathrin interaction and additional N-terminal residues present in position 300-321 may be necessary for the optimal clathrin-binding function of the motif. As for 362LEDEF366, which we found to be ineffective in binding clathrin even though it resembles a conserved clathrin-binding box, additional residues may be required to constitute a minimum functional clathrin-binding site.

The precise function of clathrin recruitment mediated by endofin-TOM1 onto endosomes remains to be established. Other studies have shown that Hrs is capable of recruiting clathrin heavy chain onto endosomes (Raiborg et al., 2001) as well as sorting of ubiquitinated proteins into clathrin-coated endosomal microdomains (Raiborg et al., 2002). Hrs has also been shown to interact directly with ubiquitin (Raiborg et al., 2002) and TSG101 (Lu et al., 2003; Katzmann et al., 2003; Pormillos et al., 2003; Bache et al., 2003b). Ubiquitin is
Membrane recruitment of clathrin by TOM1

probably the signal involved in the inward movement of proteins on the early endosomes to trigger the biogenesis of multivesicular bodies (MVB), an important step in sorting proteins to the lysosomes for degradation (Raiborg et al., 2003). TSG101 is a component of ESCRT-I (Katzmann et al., 2001). Together with ESCRT-II and ESCRT-III, ESCRT-I is the major molecular machinery involved in the sorting of proteins into the MVB. In this way, Hrs plays a key role in sorting proteins in the early endosome for delivery to the lysosome. As TOM1 is able to interact with ubiquitin (Yamakami et al., 2003) (our unpublished results), endofin-mediated endosomal recruitment of TOM1 could also contribute to the ubiquitin-recognition capability in early endosomes. Via the recruitment of clathrin as well as interaction with ubiquitylated proteins in the early endosomes, the endofin-mediated recruitment of TOM1 could play a role parallel to that of Hrs in the sorting of ubiquitinated proteins into clathrin-coated regions for subsequent delivery to the lysosome via the MVB. Consistent with this possibility, overexpression of endofin caused an accumulation of internalized EGF (Seet and Hong, 2001) as well as ubiquitin (our unpublished data) in the endosomes. Further support for this possibility may be derived from the observation that endofin seems to recruit clathrin onto early endosomes characterized by the distinct absence of the adaptor proteins AP1, AP2 and AP3. These features resembled those of a population of wortmannin-sensitive early endosomes composed of bilayer coats that contain clathrin but lack AP1, AP2 and AP3, as described in a previous report (Sachse et al., 2002). The presence of Hrs as well as other proteins involved in late stages of endosomal trafficking or targeted for lysosomal degradation in these bilayered coats suggested that the early endosomes are involved in segregating cargo proteins destined for the degradation pathway from recycled proteins (Sachse et al., 2002). It thus seems possible that multiple pathways or protein complexes on the early endosomes could exploit clathrin recruitment and ubiquitin recognition for sorting of target proteins. Alternatively, these pathways/complexes could converge on the early endosome to form a large interacting molecular network using the endosome membrane as a platform. The composition and dynamics of this intermolecular network will govern the specificity and dynamics of the sorting and trafficking events at the early endosomes.

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