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Recruitment of clathrin onto endosomes by the Tom1–Tollip complex

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Abstract

Tom1 (target of Myb 1) and its related proteins (Tom1L1/Srcasm and Tom1L2) constitute a protein family and share an N-terminal VHS (Vps27p/Hrs/Stam) domain and a following GAT (GGA and Tom1) domain, both of which are also conserved in the GGA family proteins. However, the C-terminal half is not significantly conserved between the Tom1 and GGA families or even between Tom1 and Tom1L1. We have previously shown that the GAT domain of Tom1 interacts with Tollip (Toll-interacting protein), which is associated with endosomes, to which it recruits Tom1. We here extend the previous data and show that the GAT domains of Tom1L1 and Tom1L2 also interact with Tollip, and the C-terminal regions of all the Tom1 family proteins interact with clathrin. Furthermore, when coexpressed with Tollip, all the Tom1 family proteins recruite clathrin onto endosomes. These results indicate that, in conjunction with Tollip, Tom1 family proteins play an important role in recruiting clathrin onto endosomes and suggest that they modulate endosomal functions.

Keywords: Tom1; Endosome; Clathrin; Tollip; Ubiquitin; Membrane traffic; Vesicular transport

Membrane trafficking via the endosomal system plays a pivotal role in delivering not only endocytosed proteins from the plasma membrane but also de novo-synthesized proteins from the trans-Golgi network (TGN) to appropriate intracellular destinations, including lysosomes for degradation. Various adaptor proteins have been shown to be recruited from the cytosol onto endosome membranes and regulate the endosomal trafficking. The trafficking processes to endosomal compartments are often clathrin-dependent. For example, various cargo proteins are delivered to endosomes/lysosomes via clathrin-coated vesicles from the plasma membrane and from the TGN [1,2] . Recently, clathrin coats have been found to constitute microdomains on membranes of early endosomes and multivesicular bodies (MVBs) and proposed to participate in targeting of ubiquitinated proteins to lysosomes for degradation [3–6].

GGA (Golgi-localizing, γ-adaptin ear domain homology, Arf-binding protein) family proteins (GGA1–GGA3 in mammals) and Tom1 (target of Myb 1) have recently been proposed to be implicated in the endosomal trafficking. GGAs are monomeric clathrin adaptors that regulate transport of proteins between the TGN and endosomes [7–9]. They are associated with the TGN membrane by interacting with the Arf small GTPase through their GAT (GGA and Tom1) domain, and recruit cargo proteins, such as mannose 6-phosphate receptors, by recognizing an acidic cluster-dileucine signal through their VHS (Vps27p/Hrs/Stam) domain. GGAs also recruit clathrin onto the TGN membrane through clathrin box sequences in their hinge region.

Tom1 and its related proteins (Tom1L1/Srcasm and Tom1L2) constitute a protein family and share an N-terminal VHS domain and a following GAT domain with GGAs. In contrast to GGAs, however, Tom1 interacts neither with Arf through its GAT domain nor with acidic cluster-dileucine sequences through its VHS domain, although

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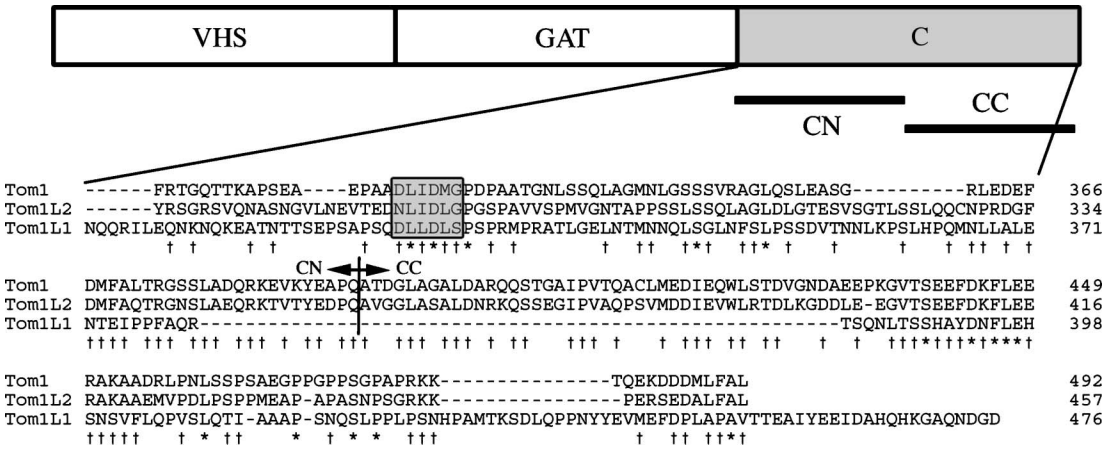

Fig. 1. Schematic representation of the structure of Tom1 and sequence alignment of the C-terminal regions of the Tom1 family proteins. Residues conserved in all members and between two of the members are indicated by asterisks and obelisks, respectively. Clathrin box sequences are boxed. Human Tom1, gi/4885637; human Tom1L1, gi/4885639; and human Tom1L2, gi/30353855.

both GGAs and Tom1 bind to ubiquitin through their GAT domains [10–12]. The C-terminal half is not significantly conserved between the Tom1 and GGA families or even between Tom1 and Tom1L1 (see Fig. 1). We [10] and others [11] have recently shown that Tom1 interacts with ubiquitinated proteins and Tollip (Toll-interacting protein) through its GAT domain. Tollip was originally identified as a negative regulator of signaling downstream of the IL-1 receptor and the Toll-like receptors [13,14]. However, its subcellular localization has not been examined. We have previously shown that Tollip is associated through its C2 domain with endosomes, to which it recruits Tom1 and ubiquitinated proteins [10]. A recent report showing an interaction between the Tollip C2 domain and phosphatidylinositol 3-phosphate (PtdIns(3)P) [15] supports our results.

In the present study, we show that all the Tom1 family proteins interact with Tollip through their GAT domain and with clathrin through their C-terminal region. Furthermore, when coexpressed with Tollip, they are associated with endosomes, to where they recruit clathrin, suggesting that the Tom1 family proteins, in conjunction with Tollip, are important modulators of endosomal functions.

Materials and methods

Plasmids. The entire coding sequences of Tom1 (gi/4885637), Tom1L1 (gi/4885639) were obtained by PCR amplification of a human liver cDNA library (Invitrogen). An IMAGE clone for human Tom1L2 (clone No. 5298847) was purchased from Invitrogen. The following cDNA fragments were amplified by PCR: Tom1 fragments for VHS + GAT (residues 1–316), GAT (residues 141–316), C-terminal region (residues 311–492), CN (residues 311–390), and CC (residues 391–492); Tom1L1 fragments for VHS + GAT (residues 1–303), GAT (residues 143–303), C-terminal region (residues 297–476), CN (residues 297–381), and CC (residues 382–476); and Tom1L2 fragments for GAT (residues 91–263), C-terminal region (residues 258–457), CN (residues 258–358), and CC (residues 359–457). These fragments were independently subcloned into the pGEX-6P or -4T vector (Amersham Biosciences) for expression in *Escherichia coli* or into the pcDNA3HAN or the pcDNA3MycN vector for expression in mammalian cells [16]. In the ΔCBox constructs of Tom1, Tom1L1, and Tom1L2, residues 321–327, 316–322, and 279-285, respectively, were deleted by PCR-mediated mutagenesis. Construction of an expression vector for HA-, FLAG-, and Myc-tagged Tollip was described previously [10]. An expression vector for HA-tagged human Hrs was a kind gift from Hiroyuki Takatsu (RIKEN, Yokohama, Japan).

Antibodies. Monoclonal mouse antibodies to clathrin heavy chain (CHC) (clone 23) and δ-adaptin (clone 18) were purchased from BD Biosciences, those to γ-adaptin (100.3), and the FLAG epitope (M2) were from Sigma, that to α-adaptin (AP6) was from Affinity Bioreagents, and that to the c-Myc epitope (9E10) was from Santa Cruz Biotechnology. Monoclonal rat antibody to the HA epitope (3F10) was from Roche Diagnostics. Alexa488- and Alexa555-conjugated secondary antibodies and peroxidase-conjugated secondary antibodies were from Molecular Probes and Jackson ImmunoResearch Laboratories, respectively.

Pull-down assays. Pull-down assays using GST-fusion proteins were performed as described previously [10,12]. Briefly, GST-fusion proteins purified from *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) were prebound to glutathione–Sepharose beads (Amersham Biosciences) and incubated with lysates from HeLa cells with or without transient expression of HA-tagged Tollip. The materials bound to the beads were subjected to immunoblot analysis using anti-CHC or anti-HA antibody and detected with ECL reagents (Amersham Biosciences).

Immunofluorescence analysis. DNA transfection and immunofluorescence analysis were performed as described previously [10,12].

Results

Interaction of Tom1 family proteins with clathrin in vitro

Previous studies showed that Tom1 interacts with clathrin mainly through a canonical clathrin box sequence, ^{321}DLIDMG^{326} (Fig. 1), within its C-terminal region [11,17]. Because the sequence appeared to be conserved in other Tom1 family members, Tom1L1 and Tom1L2 (Fig. 1), we first examined whether the C-terminal regions of the Tom1-like proteins are also able to interact with clathrin. To this end, each C-terminal fragment was purified as a GST-fusion protein and incubated with HeLa cell lysates. The bound proteins were then subjected to immunoblot analysis with anti-clathrin heavy chain (CHC) antibody. As shown in Fig. 2, upper panel, the C-terminal fragments from all the Tom1 family proteins brought down

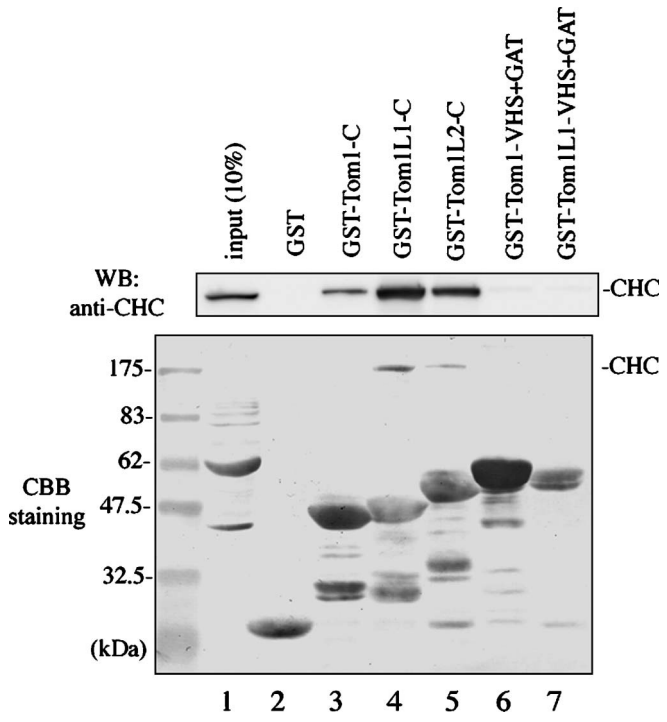

Fig. 2. Binding of clathrin to the C-terminal regions of the Tom1 family proteins in vitro. HeLa cell lysates were pulled down with GST (lane 2), or a GST-fusion protein of the C-terminal region of Tom1 (lane 3), Tom1L1 (lane 4), or Tom1L2 (lane 5) or the VHS + GAT domain of Tom1 (lane 6) or Tom1L1 that was prebound to glutathione–Sepharose beads. The bound materials were subjected to immunoblot analysis with anti-CHC antibody (upper panel). The blotted membrane was also stained with Coomassie brilliant blue (CBB) (lower panel). The position of CHC is indicated.

clathrin with the highest efficiency with the Tom1L1 fragment (lanes 3–5). It is noteworthy that the band that corresponded to CHC was visible even when the blotted membrane was stained with Coomassie brilliant blue (Fig. 2, lower panel). By contrast, the VHS + GAT domain from either Tom1 or Tom1L1 did not bring down clathrin (lanes 6 and 7, respectively). We were unable to examine the VHS + GAT domain of Tom1L2 due to the extreme insolubility of its GST-fusion protein, when expressed in *E. coli* cells.

We then investigated whether the potential clathrin box sequence in each Tom1 family protein is responsible for the clathrin binding. As shown in Fig. 3A, deletion of the clathrin box sequence (ΔCBox) from the Tom1 C-terminal region reduced extremely, although not completely abolished, its binding ability to clathrin (compare lanes 3 and 4). The results were compatible with the recent data of Seet and Hong, indicating that the Tom1 clathrin box sequence, ^{321}DLIDMG^{326}, makes a major contribution to the clathrin binding and another sequence, ^{362}LEDEF^{366}, enhances it [17]. Essentially the same results were obtained with the Tom1L2 C-terminal region; namely, deletion of the potential clathrin box sequence, ^{279}NLIDLG^{284}, extremely reduced the clathrin binding (lanes 7 and 8). By contrast, we unexpectedly found that deletion of the potential clathrin box sequence, ^{316}DLLDLS^{320} (see Fig. 1), from the Tom1L1 C-terminal region did not have any discernible effect on the clathrin binding (compare lanes 5 and 6).

We therefore divided each C-terminal region into two pieces (CN and CC, see Fig. 1) and independently

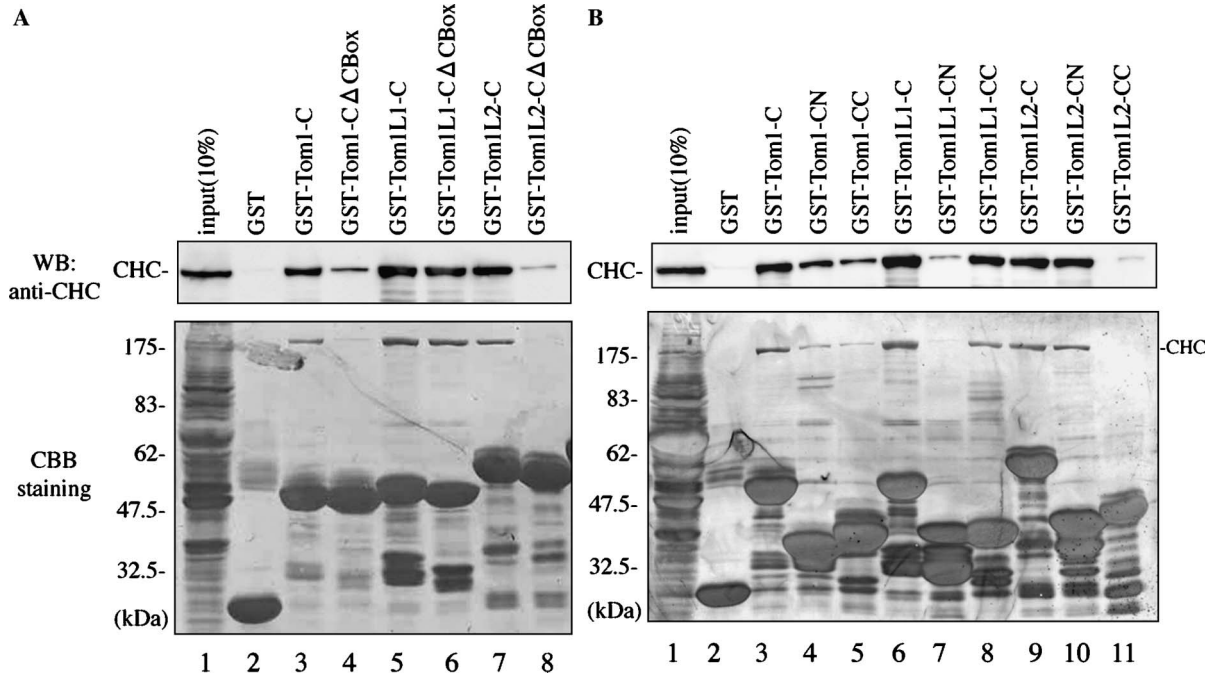

Fig. 3. Dissection of the clathrin binding regions of the Tom1 family proteins. (A) A GST-fusion protein of the C-terminal region of Tom1 (lane 3), Tom1L1 (lane 5), or Tom1L2 (lane 7), or its mutant lacking the potential clathrin box sequence (ΔCBox; see Materials and methods) (lanes 4, 6, and 8, respectively), were used to pull-down HeLa cell lysates. (B) The C-terminal regions of Tom1, Tom1L1, and Tom1L2 were divided into two fragments as schematically shown in Fig. 1. Each fragment was fused to GST and used to pull-down HeLa cell lysates. The bound materials were subjected to immunoblot analysis as described in the legend for Fig. 2.

examined their abilities to bind to clathrin (Fig. 3B). In the case of the Tom1 and Tom1L2 C-terminal regions, the CN fragment that contains a clathrin box sequence was revealed to be mainly responsible for the clathrin binding (lanes 3–5 and 9–11, respectively). On the other hand, in the case of the Tom1L1 C-terminal region, the CC fragment was found to make a major contribution to the clathrin binding (compare lanes 6–8), being compatible with the data obtained with the ΔCBox mutant (Fig. 3A; lanes 5 and 6).

Tollip recruits clathrin on endosomes through Tom1 family proteins

We and others have previously shown that the GAT domain of Tom1 interacts with Tollip, a protein involved in signaling downstream of the IL-1 receptor and the Toll-like receptors [10,11]. Furthermore, we have shown in the same study that Tollip is associated with endosomes that are positive for Hrs and EEA1, and that Tollip recruits Tom1 and ubiquitinated proteins to the endosomal structures [10]. Therefore, we first examined whether the GAT domains of Tom1L1 and Tom1L2 also interact with Tollip. As shown in Fig. 4, like the Tom1-GAT domain (lane 3), the GAT domains of Tom1L1 and Tom1L2 were also able to interact with Tollip (lanes 4 and 5, respectively). Because Tom1 and Tom1L2 share all the properties examined so far, and because the expression efficiency of Tom1L2 in mammalian cells was relatively low, we focused on Tom1 and Tom1L1 in the following experiments.

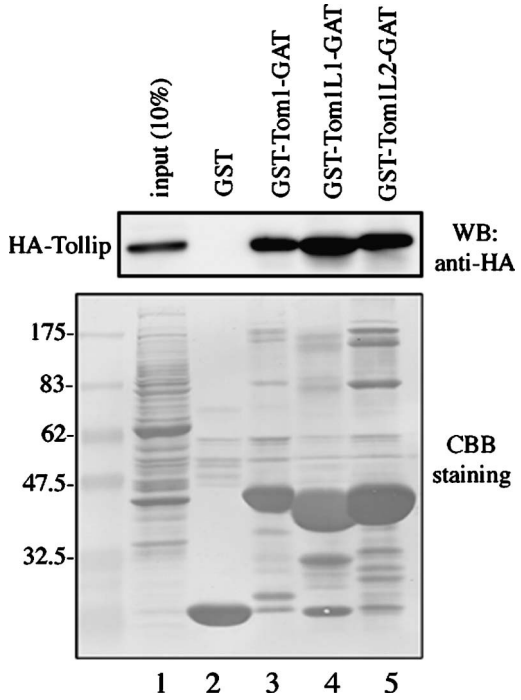

Fig. 4. Interaction of Tollip with the GAT domains of the Tom1 family proteins in vitro. Lysates of HeLa cells transiently expressing HA-tagged Tollip were pulled down with GST (lane 2), or a GST-fusion protein of the GAT domain of Tom1 (lane 3), Tom1L1 (lane 4), or Tom1L2 (lane 5) that was prebound to glutathione–Sepharose beads. The bound materials were subjected to immunoblot analysis with anti-HA antibody to detect HA-Tollip (upper panel). The blotted membrane was also stained with CBB (lower panel).

Because we have previously shown that Tollip recruits Tom1 onto endosomes [10], we reasoned that it also recruits clathrin via Tom1 and possibly via Tom1L1. When expressed alone in HeLa cells, Tom1 and Tom1L1 were largely cytoplasmic (Figs. 5A and B, respectively). In these cells, however, the distribution of clathrin was rather cytoplasmic unlike in non-transfected cells with the perinuclear, TGN-like distribution (compare cells having exogenous Tom1 (Panel A′) or Tom1L1 (Panel B′) expression with surrounding non-transfected cells). These observations make it possible that Tom1 and Tom1L1 interact with clathrin in the cytosol. Exogenously expressed Tollip associated with endosome-like structures as shown in our previous study [10] but have no discernible effect on the clathrin distribution (Panels C and C′), whereas Hrs recruited clathrin onto endosomes (Panels D and D′) as previously shown [18]. These observations suggest that Tollip is not directly involved in clathrin recruitment onto endosomes.

When coexpressed with Tollip, both Tom1 and Tom1L1 were recruited onto Tollip-positive endosomes (Panels E and E′, F and F′, respectively). Furthermore, under these conditions, clathrin was also recruited onto the Tom1- and Tom1L1-positive punctate structures (G and G′, H and H′, respectively). By contrast, coexpression of Tollip with the VHS + GAT domain of either Tom1 or Tom1L1 (namely, lacking the C-terminal region that is responsible for clathrin binding) did not lead to endosomal association of clathrin, even though the VHS + GAT domain itself was recruited onto the Tollip-positive endosome-like structures (I and I′, J and J′, respectively).

Although these observations suggested that Tom1 and Tom1L1 recruited onto endosomes were able to interact directly with clathrin, there remained a formal possibility that these proteins recruited an adaptor protein complex (AP-1, AP-2, or AP-3), which in turn recruited clathrin. To rule out this possibility, we examined the localization of the AP-1, AP-2, and AP-3 complexes in cells coexpressing Tollip and Tom1. As shown in Fig. 6, coexpression of Tollip and Tom1 did not apparently affect the distribution of AP-1 (Panel B′), AP-2 (Panel C′), or AP-3 (Panel D′), while clathrin was recruited onto the Tom1-positive endosome-like structures (A′). Essentially the same results were obtained using cells with coexpression of Tollip and Tom1L1 (data not shown). Taken together, we conclude that Tollip recruits Tom1 family proteins onto endosomes, to which the latter proteins in turn recruit clathrin.

Discussion

In the previous study [10], we showed that Tollip, which regulates signaling downstream of the IL-1 receptor and the Toll-like receptors, is associated with Hrs- and EEA1-positive endosomes and recruits Tom1 and ubiquitinated proteins. In the present study, we have extended the

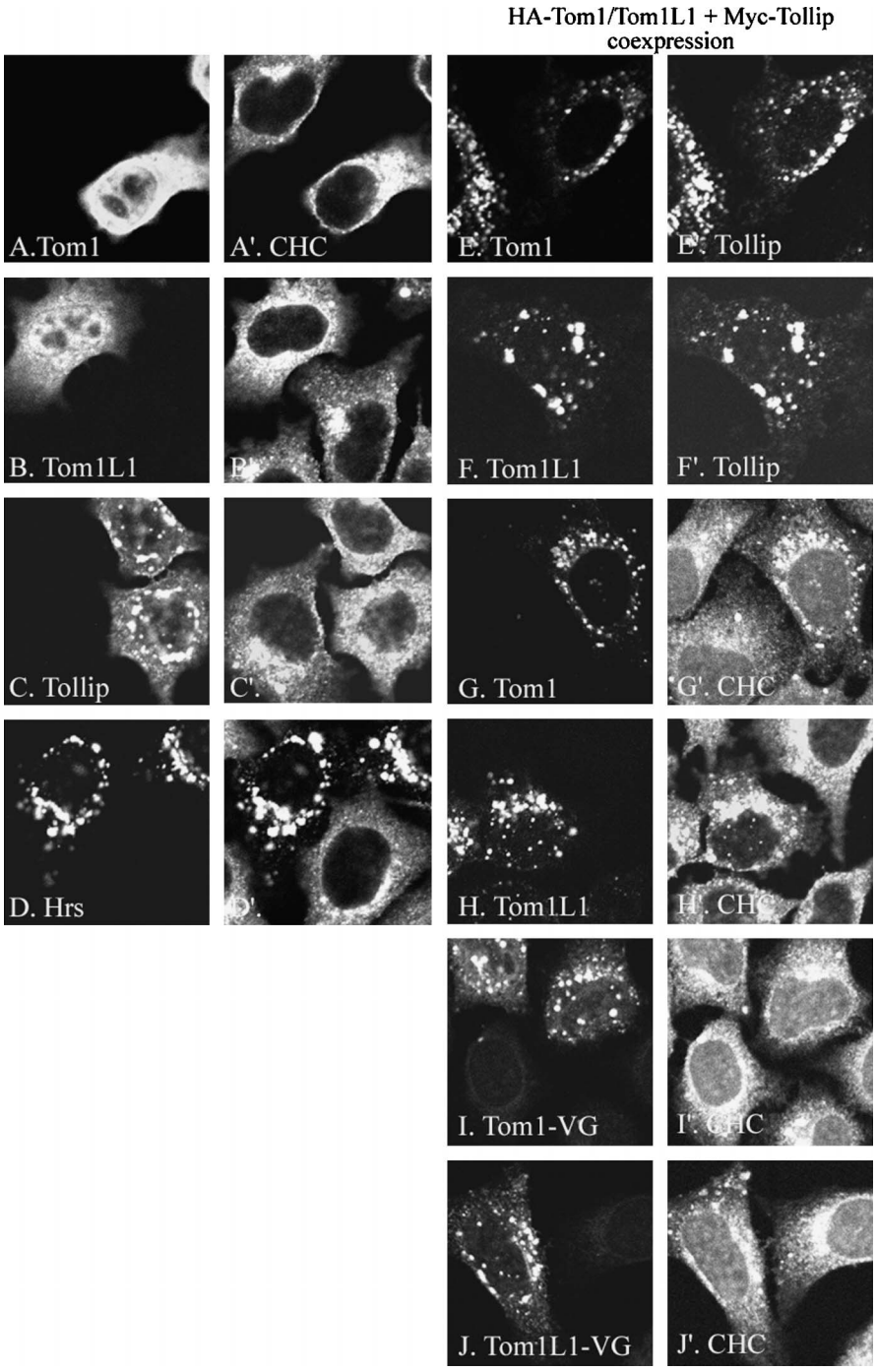

Fig. 5. Recruitment of clathrin onto Tollip-positive endosomes through the Tom1 family proteins in the cell. (A–D) HeLa cells were transiently transfected with HA-tagged Tom1 (A), Tom1L1 (B), Tollip (C), or Hrs (D) and double-stained with anti-HA (A–D) and anti-CHC (A′–D′) antibodies. (E–J) HeLa cells were transiently transfected with Myc-tagged Tollip together with HA-tagged Tom1 (E,G) or its VHS + GAT domain (I) or HA-tagged Tom1L1 (F,H) or its VHS + GAT domain (J). The cells were double-stained with anti-HA antibody (E–J) and either anti-Myc (E′,F′) or anti-CHC (G′–J′) antibody.

previous one and shown that Tollip is able to interact with all the Tom1 family proteins (Tom1, Tom1L1, and Tom1L2) and recruit clathrin onto endosomes through any Tom1 family protein. Hong and colleagues recently reported that endofin, another endosome-associated protein, also recruits clathrin via Tom1 [17]. Tollip and endofin are associated with endosomal membranes through the C2 and FYVE domains, respectively [10,19], and both domains recognize PtdIns(3)P [15,20]. However, there are two critical differences between Tollip and endofin. First, in contrast to Tollip, endofin is unable to interact with Tom1L1 and Tom1L2 [21]. Thus, Tollip appears to be more generally responsible for clathrin recruitment onto endosomes by employing any Tom1 family protein as an

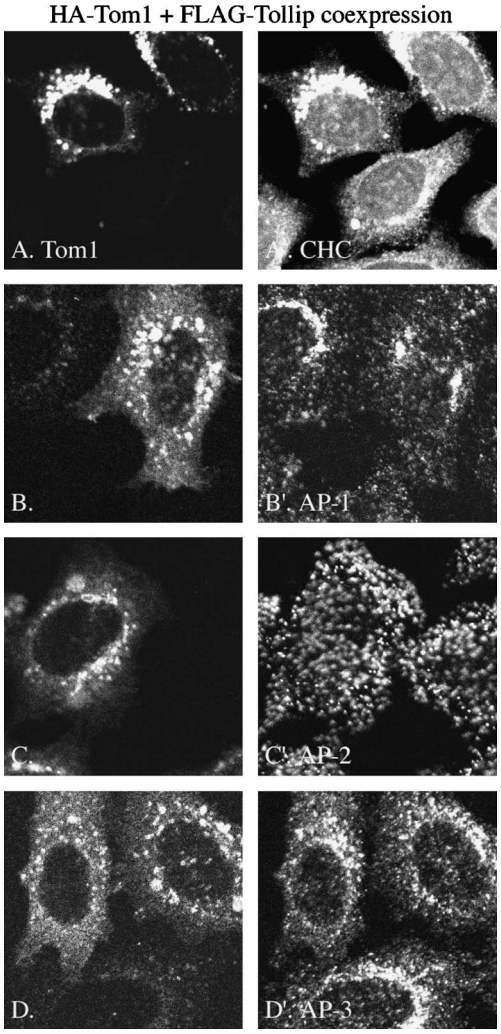

Fig. 6. Any adaptor protein complex is not recruited onto Tom1- and Tollip-positive endosomes. HeLa cells were transiently transfected with HA-tagged Tom1 and FLAG-tagged Tollip, and double-stained with anti-HA antibody (A–D) and antibody to either CHC (A′), AP-1 (B′, γ-adaptin), AP-2 (C′, α-adaptin), or AP-3 (D′, δ-adaptin).

adaptor. Second, Tollip is able to directly, as well as indirectly via Tom1 family proteins, interact with ubiquitinated proteins through its CUE domain [10] (see below). In this context, it is interesting to note that Tollip as well as other ubiquitin-binding proteins has been found to accumulate in aggregates of polyglutamine-containing proteins [22].

Among the three Tom1 family proteins, Tom1L1 appears to have the highest affinity to clathrin. Furthermore, unlike Tom1 and Tom1L2, it does not use a canonical clathrin box sequence to recruit clathrin. Because none of clathrin box variants, such as [S/D]LL and PWXXW (X stands for any amino acid) [23], is found in the Tom1L1 C-terminal region, a novel motif could be responsible for its clathrin binding. In another aspect, Tom1L1 is also distinguishable from Tom1 and Tom1L2; referred to as Srcasm (Src-activating and signaling molecule), Tom1L1 is phosphorylated by Src family kinases and modulates growth factor and Src-kinase signaling [24,25]. Taking into account the fact that, in the C-terminal region, Tom1 and Tom1L2 are similar to each other (with a ~55% amino acid identity) whereas they show very limited similarity to Tom1L1 (see Fig. 1), it is likely that Tom1L1 have somewhat different roles from those of Tom1 and Tom1L2.

The physiological relevance of clathrin recruitment onto endosomes has been proposed in the case of Hrs, which is implicated in sorting of ubiquitinated proteins into clathrin-coated microdomains [5,18]. On endosomal membranes, Hrs, in complex with Stam, binds to ubiquitinated cargo proteins, and the ESCRT (endosomal sorting complex required for transport)-I complex through its Tsg101 subunit, and serves to concentrate and localize the cargoes by interacting with the clathrin coat [26,27]. These events may trigger invagination of the endosomal membrane into the lumen to generate lumenal vesicles of the multivesicular body (MVB), which in turn fuses with lysosomes. In this context, it is interesting to note that Tollip and the Tom1 family proteins interact with ubiquitinated proteins through the CUE and GAT domains, respectively [10,11]. It is also noteworthy that Tom1L1 is able to interact with Tsg101 [28] (our unpublished results). These data together make it tempting to speculate that Tollip and the Tom1 family proteins, in concert with the ESCRT machinery, mediate sorting of ubiquitinated proteins via the MVB to lysosomes for degradation. Elucidation of the network composed of the ESCRT machinery and other accessory proteins including Tollip and the Tom1 family proteins will help to understand regulation of the sorting process.

Acknowledgments

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