Tom1 (target of Myb) is a protein of unknown function. Tom1 and its relative Tom1L1 have an N-terminal VHS (Vps27p/Hrs/Stam) domain followed by a GAT (GGA and Tom1) domain, both of which are also found in the GGA (Golgi-localizing, ADP-ribosylation factor-binding protein) family of proteins. Although the VHS and GAT domains of GGA proteins bind to transmembrane cargo proteins and the small GTPase ADP-ribosylation factor, respectively, the VHS and GAT domains of Tom1 are unable to interact with these proteins. In this study, we show that the GAT domains of Tom1 and Tom1L1 interact with ubiquitin and Tollip (Toll-interacting protein). Ubiquitin bound the GAT domains of Tom1, Tom1L1, and GGA proteins, whereas Tollip interacted specifically with Tom1 and Tom1L1. Ubiquitin and Tollip bound to an overlapping region of the Tom1-GAT domain in a mutually exclusive manner. Tom1 was predominantly cytosolic when expressed in cells. On the other hand, Tollip was localized on early endosomes and recruited Tom1 and ubiquitinated proteins. These observations suggest that Tollip and Tom1 form a complex and regulate endosomal trafficking of ubiquitinated proteins.

Ubiquitin moieties appended to proteins have been implicated in selective protein degradation by the proteasome. However, accumulating lines of evidence have shown more recently that ubiquitination, especially monoubiquitination, is a key signal in the regulation of membrane trafficking and protein degradation in lysosomes (1–5). In yeast and mammalian cells, ubiquitinated transmembrane proteins such as Ste2p and the epidermal growth factor receptor are endocytosed and sorted into luminal vesicles of the multivesicular body (MVB) (6) for degradation. In yeast, some of the biosynthetic transmembrane cargo proteins have also been shown to be delivered from the Golgi apparatus to the MVB and/or vacuole in a ubiquitination-dependent manner. The ubiquitin signal is recognized by a wide variety of cytosolic adaptor proteins that contain conserved ubiquitin-binding modules such as the UBA (ubiquitin-associated) domain, the CUE (coupling of ubiquitin to endoplasmic reticulum degradation) domain, the ubiquitin-interacting motif, the UEV (ubiquitin E2 variant) domain, and the NZF (Npl4 zinc finger) domain (Ref. 4 and references therein). These adaptor proteins often possess combinations of these modules.

Tom1 (target of Myb) was identified as a protein whose expression is induced by v-Myb (6). Tom1 and its relative Tom1L1 (also referred to as Srcasm) (7) have an N-terminal VHS (Vps27p/Hrs/Stam) domain followed by a GAT (GGA and Tom1) domain. The C-terminal half is not conserved in Tom1 and Tom1L1. The VHS domain is found in various proteins responsible for endocytic processes and signal transduction and in the GGA (Golgi-localizing, ADP-ribosylation factor-binding protein) family of proteins (8). In the case of GGA proteins, the VHS domain interacts with the ACCL (acidic amino acid cluster-LL (dileucine)) motif found in cargo receptors that cycle between the trans-Golgi network and endosomes. The GAT domain is found in GGA proteins, Tom1, and Tom1L1 (9). The GGA-GAT domain interacts with an active form of the ADP-ribosylation factor (ARF) and is responsible for membrane association of GGA proteins (reviewed in Refs. 10 and 11). In contrast to GGA proteins, the VHS domain of Tom1 or Tom1L1 does not bind to the ACCL motif, and the GAT domain of Tom1 or Tom1L1 does not bind to ARF.

To obtain a clue to the functions of Tom1 and Tom1L1, we have carried out a two-hybrid screening with their VHS and GAT domains as baits and found that the GAT domains of both Tom1 and Tom1L1 interact with ubiquitin and Tollip (Toll-interacting protein), which was originally identified by its interaction with the interleukin-1 (IL-1) receptor and Toll-like receptors (TLRs) (12) and has a CUE domain (13). Furthermore, we have shown that Tollip is localized on early endosomes, to which it recruits Tom1 and ubiquitinated proteins. While this manuscript was in preparation, Yamakami et al. (14) independently reported that Tom1 can interact with ubiquitinated proteins, Tollip, and clathrin. Our data, together with...
those of Yamakami et al., suggest that Tollip and Tom1 form a complex and regulate endosomal trafficking of ubiquitinated proteins, which might involve clathrin.

MATERIALS AND METHODS

Plasmid Construction—The entire coding sequences of human Tom1 and Tom1L1 cDNAs were obtained by PCR amplification of a human liver cDNA library (Invitrogen). cDNA fragments for Tom1 (the VHS + GAT (residues 1–316), VHS (residues 1–152), and GAT (residues 141–316) domains and the C-terminal region (residues 311–492)) and for Tom1L1 (the VHS + GAT (residues 1–303) and GAT (residues 143–303) domains) and for deletions of the Tom1-GAT domain and of human Tollip were obtained by a PCR-based strategy. Mutations of the Tom1-GAT domain resulting in defective ubiquitin binding were introduced into its cDNA using a QuikChange XL site-directed mutagenesis kit (Stratagene). For expression in Escherichia coli as a glutathione S-transferase (GST)- or His/T7-tagged protein, the cDNA fragment was subcloned into pGEX-4T2 (Amersham Biosciences) or pET-28 (Novagen), respectively. For expression in mammalian cells, the human Tom1, Tollip, or Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) cDNA fragment was subcloned into pcDNA3 (Invitrogen) with an N-terminal hemagglutinin (HA), His/FLAG, or Myc tag sequence (15). Construction of vectors for GGA-GAT domains was as described previously (16–18).

Antibodies—An antiserum was raised in a rabbit against the C-terminal 19-aminooxy acid peptide of human Tom1 conjugated to keyhole limpet hemocyanin and affinity-purified on a peptide column. The following antibodies were used in this study: mouse monoclonal antibody FK2 (raised against ubiquitin-conjugated proteins; Affiniti Research Products), mouse anti-early endosomal autoantigen-1 (EEA1) monoclonal antibody (BD Biosciences), rat anti-HA monoclonal antibody 3F10 (Roche Diagnostics), rabbit anti-GST polyclonal antibody and mouse anti-FLAG monocalonal antibody M2 (Sigma), mouse anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology), mouse anti-T7 tag monocalonal antibody (Novagen), Alexa 488-conjugated secondary antibodies (Molecular Probes, Inc.), and Cy3-conjugated and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.).

Two-hybrid Screening—A human brain cDNA library (~1.1 × 10^6 clones) was screened using the VHS + GAT domain of Tom1 or Tom1L1 as bait as described previously (16, 17).

Pull-down Assays—GST fusion proteins (~50 pmol) of the Tom1 domains were purified from E. coli BL21(DE3) cells and incubated with ubiquitin (Ub)-agarose (15 μl) or protein A-agarose (30 μl) beads (Sigma) for 1 h at room temperature in buffer A (25 mM Hepes (pH 7.4), 125 mM KCl, 2.5 mM MgOAc, 5 mM EDTA, and 1 mM dithiothreitol) containing 0.1% bovine serum albumin and 0.1% Triton X-100. The beads were then pelleted and washed three times with buffer A. Proteins associated with the beads were subjected to immunoblotting with anti-GST antibody. The blot was detected with Renaissance Plus chemiluminescent reagent (PerkinElmer Life Sciences). To examine the GAT interaction with His/T7-tagged Ub or four tandem ubiquitin units (Ub4), the Ub protein was prebound to a Pro-bond nickel chelating resin (Invitrogen) and incubated with GST-GAT (~50 pmol) for 1 h at room temperature in phosphate-buffered saline containing a protease inhibitor mixture (Complete™ EDTA-free, Roche Diagnostics). The beads were washed three times with phosphate-buffered saline, and proteins associated with the beads were subjected to immunoblotting with anti-GST antibody. To examine the GAT interaction with ubiquitinated proteins, rat liver cytosol (containing 2.5 mg of protein) was incubated with GST fusion proteins (~20 μg) of the GAT domain prebound to glutathione-Sepharose beads (Amersham Biosciences) for 1 h at room temperature in phosphate-buffered saline containing Complete™ EDTA-free mixture. Proteins associated with the beads were subjected to immunoblotting with antibody FK2. To examine the Tollip interaction with Tom1-GAT, GST-Tom1-GAT (~20 μg) prebound to glutathione-Sepharose beads was incubated with lysate from E. coli cells expressing a His/T7-tagged Tollip fragment (containing ~250 μg of protein) in buffer B (50 mM Tris- HCl (pH 7.5), 250 mM NaCl, 2.5 mM EDTA, and 1% Triton X-100) containing Complete™ for 1 h at room temperature. The beads were washed five times with buffer B. The materials bound to the beads were subjected to immunoblot analysis using anti-T7 antibody. In competition experiments, lysates from E. coli cells expressing His/T7-tagged Tollip (1–118) and from those expressing His/T7-tagged (Ub)4 were premixed and incubated with purified GST-Tom1-GAT prebound to glutathione-Sepharose beads. The materials bound to the beads were subjected to immunoblot analysis using anti-HA antibody.

Immunoprecipitation Analysis—HeLa cells grown on a 6-cm dish were transfected with the FLAG-Tom1 vector alone or in combination with the HA-tagged Tollip construct using FuGENE 6 transfection reagent (Roche Diagnostics) and cultured for 24 h. The cells were washed once with phosphate-buffered saline, scraped into 0.1 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Nonidet P-40) containing Complete™, and centrifuged at 13,500 rpm for 10 min at 4 °C in a microcentrifuge. The supernatant (containing ~200 μg of protein) was incubated with anti-HA antibody affinity beads (30 μl; Roche Diagnostics) for 2 h at 4 °C. The beads were then pelleted and washed three times with lysis buffer. The materials bound to the beads were subjected to immunoblot analysis using anti-FLAG or anti-HA antibody.

Immunofluorescence Analysis—DNA transfection and immunofluorescence analysis of HeLa cells were performed as described previously (16–18).

RESULTS

Tom1 Interaction with Ubiquitin through the GAT Domain—As described previously (16), our two-hybrid screening of a human brain cDNA library with the VHS + GAT domain of Tom1 as bait identified six ubiquitin clones. In addition, the same screening identified six Tollip clones (data not shown). Subsequent screening of the same library with the VHS + GAT domain of Tom1 identified five clones encoding Tollip, although no ubiquitin clone was obtained (data not shown). When the VHS and GAT domains were separately examined, the latter was found to be responsible for the interactions with both ubiquitin and Tollip (data not shown).
The two-hybrid interaction between Tom1 and ubiquitin was first validated by in vitro pull-down assays. The GAT domain, but not the VHS domain or the remaining C-terminal region, of Tom1 fused to GST was specifically pulled down with Ub-agarose beads (data not shown). We also examined the GAT-ubiquitin interaction using His6/T7-tagged Ub or (Ub)4. In this experiment, the GST-GAT fusion protein was pulled down with His6/T7-tagged Ub or (Ub)4 immobilized on ProBond nickel chelating beads. As shown in Fig. 1A, the Tom1-GAT domain was pulled down with both His6/T7-tagged Ub and (Ub)4 with comparable efficiencies. In addition, the GAT domain of Tom1L1 was also pulled down with the His6/T7-tagged Ub constructs. However, this was somewhat surprising for us because no ubiquitin clone was isolated by two-hybrid screening with Tom1L1 as bait and because the GAT domain of Tom1L1 was not pulled down with Ub-agarose beads (data not shown). Conformation or the conjugation state of ubiquitin might affect the interaction. To address this issue, we examined the ability of the GAT domains of Tom1 and Tom1L1 to bind to endogenous ubiquitin-conjugated proteins. To this end, rat liver cytosol was incubated with GST-GAT prebound to glutathione-Sepharose beads, and the bound materials were subjected to Western blot (WB) analysis using anti-FLAG (upper panel) or anti-HA (lower panel) antibody. In the lower panel, the asterisk indicates a nonspecific band of an unknown origin.
FIG. 4. **Competition between ubiquitin and Tollip for binding to Tom1-GAT.** GST-Tom1-GAT prebound to glutathione-Sepharose beads was incubated with a mixture of 10 μl of E. coli cell lysate expressing His6/T7-tagged Tollip-(1–118) and 0 (lane 3), 50 (lane 4), 150 (lane 5), or 500 (lane 6) μl of E. coli cell lysate expressing His6/T7-tagged (Ub)₄ or with a mixture of 50 μl of E. coli cell lysate expressing His6/T7-tagged (Ub)₄ and 0 (lane 7), 10 (lane 8), 50 (lane 9), or 250 (lane 10) μl of E. coli cell lysate expressing His6/T7-tagged Tollip-(1–118) as described under “Materials and Methods.” The bound materials were subjected to immunoblot analysis using anti-T7 tag antibody. In lanes 1 and 2, 2 μl of cell lysates expressing His6/T7-tagged Tollip-(1–118) and (Ub)₄, respectively, were loaded.

FIG. 5. **Co-localization of Tom1 and Tollip on endosomal structures.** HeLa cells were transfected with the expression vector for HA-tagged Tollip (A, D, D', and D''), HA-tagged TollipΔTBD (E, E', and E''), or FLAG-tagged Tom1 (B) alone or cotransfected with the HA-Tollip and FLAG-Tom1 vectors (C, C', and C''). The cells were stained with anti-FLAG (A) or anti-HA (B) antibody or with both antibodies (C, C', and C'') or with anti-HA and anti-Tom1 antibodies (D, D', D'', E, E', E'', and E''). Merged images are shown in C'', D'', and E''. Red indicates Tollip, and green indicates Tom1.
subjected to immunoblot analysis using monoclonal antibody FK2, which is able to react with the ubiquitin moiety of ubiquitin-conjugated proteins, but not with free ubiquitin (19, 20). As shown in Fig. 1B (lane 3), the GAT domain of Tom1 efficiently pulled down ubiquitin-conjugated proteins detectable by antibody FK2, compatible with the data presented by Yamakami et al. (14). The Tom1L1-GAT domain also pulled down ubiquitin-conjugated proteins, albeit much less efficiently compared with the Tom1-GAT domain (Fig. 1B, lane 7).

X-ray crystallographic studies carried out by us (21) and by others (22–24) have revealed that the GAT domain of GGA1 has an α-helical fold composed of two subdomains. The N-terminal GAT (referred to as N-GAT) subdomain forms a helix-loop-helix structure composed of helix α9 (helix numbering is according to Ref. 21) (Fig. 2) and an N-terminal half of the long helix (helix α1) and is responsible for binding to the GTP-bound form of ARF (reviewed in Ref. 11). On the other hand, the C-terminal GAT (referred to as C-GAT) subdomain forms a helix bundle composed of the remainder of helix α1 and all of helices α2 and α3 (Fig. 2). Furthermore, our recent study (16) has shown that, in the case of the GGA3-GAT domain, the C-GAT subdomain, especially Leu276, Leu280, and Asp284 (indicated by red arrowheads in Fig. 2) exposed on one side of helix α3, makes a major contribution to the interaction with the so-called Ile44 patch of ubiquitin. The GAT domains of GGA proteins resemble those of Tom1 and Tom1L1 within the C-GAT subdomain, but not significantly within the N-GAT subdomain (Fig. 2), suggesting that the C-GAT subdomains of Tom1 and Tom1L1 are also responsible for ubiquitin binding. Our mutational experiment supported this expectation; deletion of helix α3 (GATΔC1) (Fig. 1B, lane 4), but not of the N-terminal region (data not shown), of the Tom1-GAT domain abrogated the binding to ubiquitinated proteins. Furthermore, mutations of Leu280 (Fig. 1B, lane 5) and Asp284 (lane 6), which correspond to Leu276 and Asp284, respectively, in GGA3, also abrogated the binding.

Interaction between the Tom1-GAT Domain and Tollip—We then focused upon the GAT-Tollip interaction. Tollip interacts with the cytoplasmic domains of the IL-1 receptor and TLRs and regulates their downstream signaling (12, 25, 26). Furthermore, it has a CUE domain (see Fig. 8) that can interact with ubiquitin (13, 27).

The CUE domain is found in proteins involved in endosomal functions, such as yeast Vps9p (13, 27). We first validated the two-hybrid interaction between the Tom1-GAT domain and Tollip by a pull-down assay. Because a GST fusion protein of full-length Tollip was insoluble in E. coli cells, we divided it into two pieces. The N-terminal half (residues 1–118), but not the C-terminal half containing the CUE domain (residues 117–274), of Tollip pulled down the Tom1-GAT domain (data not shown). Further analysis using a series of deletions indicated that an N-terminal region covering a Glu-rich stretch of Tollip (residues 16–45) (Fig. 2) was minimally required for the interaction with the Tom1-GAT domain (data not shown), in good agreement with the region determined by Yamakami et al. (14). We then examined whether the GAT domain derived from other proteins could also interact with Tollip. As shown in Fig. 3A, Tollip (1–118) was efficiently pulled down with the GAT domain derived from Tom1L1, but much less efficiently with the GGA-GAT domains.

Next, we attempted to delimit the Tollip-interacting region of the Tom1-GAT domain. As shown in Fig. 3B (lane 7), a GAT construct lacking the entire N-GAT subdomain (GATΔN2) (see Fig. 2) retained the ability to interact with Tollip. In contrast, deletion of helix α3 (GATΔC1) (Fig. 3B, lane 4) resulted in an extremely feeble Tollip interaction. These results suggested that the region of the C-GAT subdomain responsible for the interaction with Tollip overlapped with the ubiquitin-binding region (see Fig. 8). However, our data indicated that the interface of the C-GAT subdomain responsible for the Tollip interaction differed from that for ubiquitin binding: the GAT mutants defective in ubiquitin binding, L285R and D289G (Fig. 2), resulted in an extremely feeble Tollip interaction. These results suggested that the region of the C-GAT subdomain responsible for the interaction with Tollip overlapped with the ubiquitin-binding region (see Fig. 8). However, our data indicated that the interface of the C-GAT subdomain responsible for the Tollip interaction differed from that for ubiquitin binding: the GAT mutants defective in ubiquitin binding, L285R and D289G (Fig. 3B, lanes 8 and 9, respectively), could interact with Tollip with efficiencies comparable with that of the wild-type GAT domain (lane 3).

We then examined whether the interactions of the GAT domain with ubiquitin and with Tollip occur simultaneously or in a mutually exclusive manner. To this end, a mixture of a constant amount of Tollip and varying amounts of (Ub)4 or vice versa was incubated with a limited amount of GST-Tom1-GAT immobilized on glutathione-Sepharose beads, and the proteins bound to the beads were examined. As shown in Fig. 4, as the
amount of (Ub)_4 added to the mixture was increased, the amount of Tollip bound to the GAT domain decreased (lanes 3–6), and as the amount of Tollip was increased, the amount of (Ub)_4 bound to the GAT domain decreased (lanes 7–10). The data indicate that the interactions of Tollip and ubiquitin with the Tom1-GAT domain are mutually exclusive.

To examine whether the Tom1-Tollip interaction indeed takes place in the cell, we performed the following experiment. First, FLAG-tagged Tom1 was coexpressed with HA-tagged Tollip or its deletion mutant lacking the Tom1-binding domain (TollipΔTBD, residues 45–274), TollipΔCUE (residues 1–230), or TollipΔTBD/ΔCUE (residues 45–230) with HA-Tom1 and Myc-Tollip vectors and double-stained with anti-HA antibody and antibody FK2 (A–F). Merged images are shown in A′–F′. Red indicates HA-Tom1 or HA-Tollip, and green indicates antibody FK2.

**Fig. 7.** Accumulation of ubiquitinated proteins on Tollip-positive endosomal structures. HeLa cells were transfected with the expression vector for HA-tagged Tom1 (A, A′, and A″) or HA-tagged wild-type Tollip (C, C′, and C″), TollipΔTBD (residues 45–274) (D, D′, and D″), TollipΔCUE (residues 1–230) (E, E′, and E″), or TollipΔTBD/ΔCUE (residues 45–230) (F, F′, and F″) or cotransfected with the HA-Tom1 and Myc-Tollip vectors (B, B′, and B″) and double-stained with anti-HA antibody (A–F) and antibody FK2 (A′–F′). Merged images are shown in A′–F′.

**Tollip Recruits Tom1 onto Endosomes**—In an attempt to examine the subcellular localization of Tom1 and Tollip and to further examine the Tom1-Tollip interaction in the cell, we performed immunofluorescence analyses. Although Tollip has been shown to be a negative regulator of signaling downstream of the IL-1 receptor and TLRs (12, 25, 26) and to interact with ubiquitin through its CUE domain (27), its subcellular localization has not been explored. When expressed in HeLa cells, HA-Tollip was localized on punctate structures distributed throughout the cytoplasm (Fig. 5A). The punctate staining for Tollip significantly, albeit partially overlapped with that for an early endosomal marker, EEA1 (Fig. 6, A, A′, and A″), but not significantly with that for the cation-independent mannose 6-phosphate receptor (data not shown), which cycles between the trans-Golgi network and late endosomes. These observations suggest that Tollip localizes on part of the population of early endosomes. Because the partial co-localization of EEA1 with Tollip was reminiscent of the partial co-localization of EEA1 with
Hrs (28, 29), which is known to sort ubiquitinated proteins to the MVB/lysosomes by virtue of its ubiquitin-interacting motif (30), we coexpressed HA-Hrs and Myc-Tollip in HeLa cells and examined their localization. As shown in Fig. 6 (B, ′B, and ″B), the Tollip-positive structures partially overlapped with the Hrs-positive structures. Thus, a significant portion of Tollip appears to be associated with early endosomes and MVBs.

Tollip mutants lacking the TBD (TollipΔTBD) (Fig. 7D), the CUE domain (TollipΔCUE) (Fig. 7E), and both domains (TollipΔTBD/ΔCUE) (Fig. 7F) all retained the ability to associate with the endosomal structures. These observations indicate that the association of Tollip with endosomes does not depend on its binding to Tom1 or ubiquitin. The C2 domain or another region may determine the endosomal localization, although we did not further explore this issue in this study.

On the other hand, Tom1 was predominantly cytoplasmic when expressed alone (Fig. 5B). In striking contrast, Tom1 was completely co-localized with Tollip on the endosome-like structures when these two proteins were coexpressed (Fig. 5, C, ′C, and ″C). We then raised a polyclonal antiserum against Tom1 to examine the localization of endogenous Tom1. The affinity-purified antiserum could detect a specific, albeit weak band for Tom1 in immunoblot analysis (data not shown). In immunofluorescence analysis, however, the antiserum did not stain distinctive subcellular structures, although faint punctate structures were often observable throughout the cytoplasm (for example, see the cell marked by an asterisk in Fig. 5, D′ and D″). In striking contrast, endogenous Tom1 was co-localized with exogenously expressed HA-Tollip on the punctate structures (Fig. 5, D, ′D, and ″D). The punctate Tom1 staining was not observed when HA-TollipΔTBD was coexpressed (Fig. 5, E, ′E, and ″E), indicating that the Tom1-Tollip interaction is essential for the endosomal localization of Tom1. On the basis of these observations, we conclude that Tollip can associate with early endosomes, to which it, in turn, recruits Tom1.

Recruitment of Ubiquitin-conjugated Proteins by Tollip and Tom1 in the Cell—Our data presented so far suggest that Tom1 and Tollip can independently interact with ubiquitin-conjugated proteins through the GAT and CUE domains, respectively. In cells that exogenously express Tollip, accumulation of ubiquitin-conjugated proteins detected with antibody FK2 was found predominantly on the Tollip-positive endosomal structures, whereas in non-transfected control cells, antibody FK2 staining was found largely in the nucleus and faintly in the cytoplasm (compare cells with and without HA-Tollip expression in Fig. 7, C, ′C, and ″C), indicating that Tollip can recruit ubiquitinated proteins in the cell. In cells that overexpressed both Tollip and Tom1, the endosomal staining for Tom1 overlapped with that for ubiquitinated proteins (Fig. 7, B, ′B, and ″B). In contrast, in cells overexpressing Tom1 alone, a reproducible, albeit slight enhancement of cytoplasmic staining with antibody FK2 was observed (compare cells with and without exogenous Tom1 expression in Fig. 7, A, ′A, and ″A), suggesting that Tom1 is also capable of binding ubiquitinated proteins in the cell.

TollipΔTBD was localized on endosomes and able to recruit ubiquitinated proteins (Fig. 7, D, ′D, and ″D), indicating that neither the ubiquitin recruitment nor the endosomal association of Tollip is dependent on the Tom1-Tollip interaction. To our surprise, TollipΔCUE, which lacks the CUE domain responsible for ubiquitin binding, could also recruit ubiquitin-conjugated proteins onto the endosomal structures (Fig. 7, E, ′E, and ″E). In contrast, another Tollip mutant lacking both the TBD and the CUE domain (TollipΔTBDΔCUE) still retained the ability to associate with endosomal structures, yet failed to recruit ubiquitinated proteins (Fig. 7, F, ′F, and ″F). A straightforward explanation for these observations is that, through its TBD, TollipΔCUE recruited endogenous Tom1, which, in turn, recruited ubiquitin-conjugated proteins through its GAT domain. However, this appears to be incompatible with our data showing that interactions of ubiquitin and Tollip with the Tom1-GAT domain are mutually exclusive (Fig. 4). Another possible explanation is related to the finding of Zhang and Ghosh (25) showing that Tollip can form a homo-oligomer. We could reproduce their data (data not shown). They further showed that a Tollip mutant lacking either the N-terminal region (corresponding to the TBD) or the C-terminal region (corresponding to the CUE domain) retains the ability to self-oligomerize and that deletion of both regions abolishes this ability. Therefore, it is tempting to speculate that TollipΔTBD and TollipΔCUE can recruit endogenous Tollip, which, in turn, recruits ubiquitinated proteins through its CUE domain.

**DISCUSSION**

In this study, we have shown that Tom1 interacts with Tollip and ubiquitin and revealed the molecular basis for the interactions. In support of our results, Yamakami et al. (14) reported Tom1 interactions with Tollip and ubiquitin while this manu-
script was in preparation. In addition, we have shown that Tollip localizes on early endosomes and recruits Tom1 and ubiquitin-conjugated proteins to these compartments. The relationship of the interactions revealed in this study and in the study of Yamakami et al. (14) is schematically shown in Fig. 8. Tom1 interacts with ubiquitinated proteins and Tollip through the C-GAT subdomain in a mutually exclusive fashion. On the other hand, Tollip interacts with Tom1, endosomes, and ubiquitinated proteins through the N-terminal TBD including the Gln-rich stretch, the central region including the C2 domain, and the C-terminal CUE domain, respectively.

The GAT domain is found in GGA proteins, Tom1, and Tom1L1. The C-GAT subdomain of GGA1 has been shown to form a compact three-helix bundle by x-ray crystallographic studies carried out by us (21) and by others (22–24). It was reasoned that the C-GAT subdomain plays a common role in GGA proteins, Tom1, and Tom1L1 because this subdomain is highly conserved among these proteins (Fig. 2). In our previous (16) and present studies, we have indeed shown that the C-GAT subdomains of GGA3 and Tom1 bind ubiquitin based on the same structural results. On the other hand, we have shown that the C-GAT subdomains of Tom1 and Tom1L1, but not those of GGA proteins, can interact with Tollip, suggesting that residues of the C-GAT subdomain of Tom1 that are not conserved in GGA proteins are responsible for the Tollip interaction. Our data indicating that the Tom1-GAT domain uses distinct interfaces to interact with ubiquitin and Tollip are compatible with this speculation.

In contrast to the C-GAT subdomain, the N-GAT subdomain is not significantly conserved between GGA proteins and Tom1 and Tom1L1 (Fig. 3). The N-GAT subdomain of GGA proteins, which forms a helix-loop-helix structure (21, 22, 24), binds the small GTPase ARF (18, 21, 31), whereas the N-GAT subdomain of Tom1 cannot bind ARF. Furthermore, the preceding VHS domain of Tom1 or Tom1L1 cannot bind the ACLL sequences found in the cytoplasmic tails of various transmembrane cargo proteins, although this domain is significantly conserved in GGA proteins, Tom1, and Tom1L1 (17, 32). It seems therefore likely that the N-GAT subdomain and the VHS domain of Tom1 and Tom1L1 play roles distinct from those for the corresponding domains of GGA proteins.

The C-terminal half-region is not conserved even between Tom1 and Tom1L1. Yamakami et al. (14) reported that the C-terminal region of Tom1 interacts with clathrin. Thus, Tom1 is similar to Hrs not only in having a VHS domain, but also in its ability to interact with ubiquitinated proteins and clathrin (29, 30). It is therefore possible that, like Hrs (30, 33), Tom1 is also involved in sorting of ubiquitinated proteins into clathrin-coated microdomains of early endosomes or MVBs. While this manuscript was in preparation, Seet et al. (34) reported that Tom1 interacts with an endosomal protein, Endofin, through a region following the GAT domain. Although little is currently known about the role of Endofin, it associates with endosomes through its phosphatidylinositol 3-phosphate-binding FYVE domain. Taken together with the data that Tom1 (~60 kDa) and Tollip (~30 kDa) in HEK293 cell extracts are coeluted with a molecular mass of ~350 kDa on a gel filtration column (14), it is possible that Tom1 is included in a large heteromeric complex that contains Tollip, Endofin, and ubiquitin-conjugated proteins and regulates endosomal functions. On the other hand, the C-terminal region of Tom1L1 has been reported to be capable of binding various Src homology-3 domain-containing proteins through its polyproline motif (7). Thus, the C-terminal regions of Tom1 and Tom1L1 appear to be related to their specific functions.

The N-terminal TBD and the C-terminal CUE domain of Tollip bind Tom1 and ubiquitin, respectively, and the central region including the C2 domain is responsible for association with endosomal membranes. Because the C2 domain is known to bind in a Ca\(^{2+}\)-dependent manner not only to phospholipids, but also to proteins (reviewed in Ref. 35), Tollip might associate with endosomal membranes through its C2 domain. Many adaptor proteins possessing ubiquitin-binding module(s) associate with endosomes and have been shown to be involved in sorting of ubiquitinated cargos to luminal vesicles of MVBs, determining the delivery of the cargo proteins to lysosomes for degradation (reviewed in Refs. 1–5). Furthermore, these ubiquitin-binding proteins often form complexes to achieve their functions. For example, a ubiquitin-interacting motif-containing protein, Hrs, and its yeast ortholog, Vps27p, which both contain a VHS domain, are complexed to other ubiquitin-interacting motif-containing proteins, Eps15 and Stam/Ise1p (36–39). This complex formation can serve to enhance the affinity for ubiquitin-conjugated proteins because each ubiquitin-binding module binds ubiquitin with low affinity (5). This might hold true for Tollip, as it also associates with endosomes, self-oligomerizes, and forms a complex with Tom1.

Tollip was originally identified as a modulator of IL-1 receptor signaling (12) and later shown to be involved in signaling downstream of TLR2 and TLR4 (25, 26). In a proposed model for Tollip function (12), it forms a complex with IL-1 receptor-associated kinase-1 (IRAK-1) under resting conditions, and the Tollip-IRAK-1 complex transiently associates with the receptors upon stimulation with agonists such as IL-1\(\beta\) and lipopolysaccharide. After association with the receptors, IRAK-1 dissociates from Tollip as it phosphorylates itself and Tollip. In turn, activated IRAK-1 phosphorylates downstream molecules to fulfill its function. Overexpressed Tollip has been reported to inhibit signaling downstream of the IL-1 receptor and TLRs by suppressing the kinase activity of IRAK-1 (12, 25, 26). It is therefore of great interest whether Tom1 is also involved in this signaling process. However, our preliminary analyses so far have failed to show such an involvement. First, we could not co-immunoprecipitate IRAK-1 with Tom1 under a variety of experimental conditions. This suggests that IRAK-1 and Tom1 are not simultaneously included in a Tollip-containing complex. Second, upon stimulation with IL-1\(\beta\), we could not detect translocation of Tollip to the Tom1-positive plasma membrane or that of the IL-1 receptor to Tollip-positive endosomes. It is thus likely that Tom1 functions irrespective of IL-1 signaling, although further experiments will be required to address this issue.

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Yohei Katoh, Yoko Shiba, Hiroko Mitsuhashi, Yuko Yanagida, Hiroyuki Takatsu and Kazuhisa Nakayama

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