Tom1, a VHS Domain-containing Protein, Interacts with Tollip, Ubiquitin, and Clathrin*

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The gene for Tom1 was initially identified as a specific target of the oncornogen v-myb. The Tom1 protein belongs to the VHS domain-containing protein family, and it has a GAT domain in a central part as well as an N-terminal VHS domain. VHS domain-containing proteins, including Hrs/Vps27, STAM, and GGA proteins, have been implicated in intracellular trafficking and sorting, but the role of Tom1 has not yet been elucidated. In this study, we found that Tom1 binds directly with ubiquitin chains and Tollip, which was initially isolated as a mediator of interleukin-1 signaling and has a capacity to bind ubiquitin chains. Gel filtration and subsequent Western blot analysis showed that endogenous Tom1 associates with Tollip to form a complex. In addition, Tom1 was found to be capable of binding to clathrin heavy chain through a typical clathrin-binding motif. Fluorescence microscopic analysis revealed that green fluorescent protein–Tom1 was localized predominantly in the cytoplasm, whereas its mutant with deletion of the clathrin-binding motif had a diffuse localization throughout the cell. Thus, we propose that a Tom1-Tollip complex functions as a factor that links polyubiquitinated proteins to clathrin.

The endosome system is involved in the trafficking and sorting of plasma membrane proteins that have undergone endocytosis and newly synthesized proteins for which the final destination is the lysosome (1, 2). Various adaptor proteins mediate the trafficking and sorting pathways in the endosome system (3), and ubiquitin has recently been shown to function as a sorting signal in this system (4–6). Several proteins containing a VHS1 domain have been shown to participate in the intracellular trafficking and sorting pathways (7). The VHS domain is an ~140-residue domain, the name of which is derived from its presence in Vps27, Hrs, and STAM and which is present at the N termini of proteins. VHS domain-containing proteins can be divided into four groups (7). The first group, which includes STAM, EAST (epidermal growth factor receptor-associated protein with Src homology 3 and TAM domains), and Hbp (Hrs-binding protein), consists of proteins that each have Src homology 3 and immunoreceptor tyrosine-based activation motif domains and a ubiquitin-interacting motif (UIM) that can bind ubiquitin (5) as well as an N-terminal VHS domain. Proteins in the second, including Hrs and Vps27, each contain a VHS domain, an FYVE (Fab1b, YOTB, Vac1p, and EEA1) domain that can bind to membrane lipid, a coiled-coil domain that can bind with STAM, a UIM, and a clathrin-binding motif (clathrin box) that is recognized by clathrin (8, 9). The third group consists of GGA proteins that each contain a VHS domain, a GAT (for GGA and Tom1) domain that shows homology to the GAT domain of Tom1 and binds with ARF (a low molecular weight GTP-binding protein), a flexible hinge region that contains clathrin boxes, and a C-terminal γ-adaptin ear domain with homology to the ear domain of γ-adaptin. The fourth group consists of proteins with an N-terminal VHS domain alone or with other additional domains.

Among the VHS domain-containing proteins, STAM and Hrs bind with each other through their coiled-coil domains to form a complex, and this complex is capable of binding ubiquitin through their UIMs and functions as a sorting factor for ubiquitinated membrane proteins at the endosome (10–14). In addition, GGA proteins recognize the acidic cluster-dileucine motifs present in mannose 6-phosphate receptors through their VHS domains and have been implicated in the sorting of these receptors at the trans-Golgi network (15–19). By means of interactions with these sorting receptors, ARF, and clathrin, GGA proteins play critical roles in the transport of soluble and membrane-bound proteins from the trans-Golgi network to the endosome/lysosome. Thus, the above VHS domain-containing proteins have been proposed to function as adaptor proteins in the intracellular trafficking and sorting of plasma membrane proteins and newly synthesized proteins, but the functions of other VHS proteins such as Tom1 have not yet been elucidated.

The gene for Tom1 was initially identified as a specific target of retroviral oncornogen v-myb (20). This protein has an N-terminal VHS domain (21), followed by a short GAT domain (15). The crystal structures of the VHS domains of Tom1 (22), Hrs (23), and GGA proteins (24, 25) have been determined, and their structures were found to be composed of a right-handed super helix of eight helices. Although their global structures are similar, the VHS domains of GGA1 and GGA3, but not those of Tom1 and Hrs, have a specific recognition pocket for the acidic cluster-dileucine sequence in the cytoplasmic tail of the mannose 6-phosphate receptor (24, 25). The crystal struc-
tured of the GAT domain of GGA1 has recently been reported to be composed of a three-helix bundle with a long N-terminal helical extension, and this helical extension, which is not conserved in the GAT domain of Tom1, was found to be indispensable for the association of GGA1 with ARF (26–28). Thus, the structure-function relationships in the VHS and GAT domains of GGA proteins have been extensively elucidated, but those of Tom1 have not yet been determined.

To determine the functions of Tom1, we searched for proteins that interact with Tom1; by yeast two-hybrid screening, we identified two proteins, Tollip, which has been isolated as a component of the interleukin-1 signaling pathway (29), and ubiquitin, as Tom1-binding proteins. In this study, we found that Tom1 directly associates with Tollip to form a complex and that Tom1, as well as Tollip, is capable of directly binding with polyubiquitin chains. We also found that Tom1 binds with clathrin heavy chain through its clathrin box. Based on the above findings, we propose that Tom1 functions as a factor that links polyubiquitinated proteins to clathrin.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney (HEK) 293T, HEK293, and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). Transfection was performed using FuGENE 6 (Roche) according to the manufacturer’s protocol.

Plasmid Construction—The open reading frames of mouse Tom1 and Tollip both containing EcoRI and SalI sites were amplified by polymerase chain reaction (PCR). The PCR fragments were subcloned into the pGEM-T vector (Promega). All constructs were verified by DNA sequencing. Truncated mutants of Tom1 and Tollip were generated by PCR. To generate the expression plasmids, the respective PCR fragments that had been digested with EcoRI and SalI were inserted into the EcoRI and SalI sites of mammalian expression vectors, pCI-neo-T7 (Promega) and pSF1, that had been generated by inserting a fragment encoding three copies of Flag epitope amplified by PCR from the p3xFlag vector (Sigma) into the pcDNA3.1/Myc-His(−)6 vector (Invitrogen). The pSF1 vector was prepared by Dr. Y. Saeki in our laboratory.

For yeast two-hybrid screening, the PCR fragment encoding the C-terminally truncated mutant of Tom1, Tom1-(1–310), was subcloned into the pGBK7 vector (Clontech). For expressing GST fusion proteins, the PCR fragments encoding full-length Tom1, Tom1-(180–492), and full-length Tollip were subcloned into the pGEX6P1 vector (Amersham Biosciences).

Yeast Two-hybrid Screening—Saccharomyces cerevisiae AH109 cells transformed with the pGBK7-Tom1-(1–310) plasmid and an NIH3T3 MATCHMAKER cDNA library in the pACT2 vector (Clontech) were plated on media that lacked tryptophan, leucine, and histidine and had been supplemented with 20 mM 3-amino-1,2,4-triazole (Sigma). Approximately 1.4 × 10^6 yeast transformants were screened according to the instructions from the manufacturer. cDNAs derived from positive clones were extracted using a Yeast Plasmid Minipreparation kit (ZymoResearch) and subjected to nucleotide sequence determination.

Isolation of Recombinant Proteins and Preparation of Polyclonal Antibodies—Escherichia coli BL21 (DE3) cells transformed with the pGEX6P1-Tollip, pGEX6P1-Tom1, and pGEX6P1-Tom1-(180–492) expression plasmids were cultured overnight at 37 °C in LBA medium (Lucas Bertani medium supplemented with 50 μg/ml ampicillin), transferred to a 20-fold volume of LBA medium containing 37 °C C for 2 h. After the cells had been chilled in water, isopropyl-1-thio-β-D-galactopyranoside was added to the cells at the final concentration of 0.1 mM and the cells were incubated at 20 °C for 24 h. The cells were then harvested and lysed by Y-PEL (Pierce) supplemented with 1 mM dithiothreitol and a protease inhibitor mixture (Roche Molecular Biochemicals). After the lysate had been centrifuged at 15,000 rpm for 20 min, the resulting supernatant was mixed with glutathione-immobilized agarose beads (Amersham Biosciences) and rotated at 4 °C for 2 h, and the beads were extensively washed with buffer A (20 mM Tris- HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol, and the complete protease inhibitor mixture). GST fusion proteins were then eluted from the beads with 50 mM Tris- HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol, and the complete protease inhibitor mixture. GST fusion proteins were then eluted from the beads with 50 mM Tris- HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol, and the complete protease inhibitor mixture.

Alternatively, PreScission protease (Amersham Biosciences) was added to the beads and the mixture was incubated at 4 °C overnight to allow the protease to cleave the specific site between GST and the respective protein. The proteins thus formed were then eluted from the beads and purified by fast protein liquid chromatography on a Mono-Q column (Amersham Biosciences). To prepare anti-Tom1 and anti-Tollip antibodies, the thus purified Tom1-(180–492) and Tollip, respectively, were emulsified with Titer Max Gold (CytRx) and injected subcutaneously into rabbits followed by several booster injections.

Immunoprecipitation and Western Blotting—HEK293T cells were treated with 50 μM MG132 (Peptide Institute, Osaka, Japan) for 6 h to inhibit proteosomal degradation of polyubiquitiinated proteins, washed with ice-cold phosphate-buffered saline, and lysed with buffer B containing 50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and the protease inhibitor mixture. After centrifugation, the resulting supernatant was incubated with protein G- or protein A/G-immobilized agarse beads (Santa Cruz) that had been preloaded with anti-Tom1 antibody, and the immunocomplex produced was eluted with 0.1M glycine HCl, pH 3.0, separated by SDS-PAGE and then to Western blotting with anti-Tom1, anti-GST, or anti-ubiquitin antibody. For yeast two-hybrid screening, the PCR fragment encoding the C-terminally truncated mutant of Tom1, Tom1-(1–310), was subcloned into the pGBK7 vector (Clontech). For expressing GST fusion proteins, the PCR fragments encoding full-length Tom1, Tom1-(180–492), and full-length Tollip were subcloned into the pGEX6P1 vector (Amersham Biosciences).

For Western blotting, the whole cell lysate and the immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were immunoblotted with anti-Tom1, anti-Tollip, anti-GST (Santa Cruz), anti-T7 (Novagen), anti-Flag M2 (Sigma), anti-ubiquitin (Novagen), anti-polyubiquitin (Santa Cruz), (30), and anti-clathrin heavy chain (BD Transduction Laboratories) antibodies and incubated with horseradish peroxidase-conjugated antibody against mouse or rabbit immunoglobulin (Amersham Biosciences), followed by detection with ECL immunoblotting detection reagents (Amersham Biosciences).

Gel Filtration—HEK293 cells were lysed with buffer C and centrifuged at 15,000 rpm for 30 min. The resulting supernatant (200 μl) was applied on a column (1 × 30 cm) of Superdex 200 HR (Amersham Biosciences) that had been equilibrated with buffer B. Fractions (each 400 μl) were collected, and the aliquots were subjected to SDS-PAGE followed by Western blotting with anti-Tom1 and anti-Tollip antibodies.

Alternatively, the molecular mass was estimated by gel filtration—HPLC (Amersham Biosciences). To prepare anti-Tom1 and anti-Tollip antibodies, the thus purified Tom1-(180–492) and Tollip, respectively, were emulsified with Titer Max Gold (CytRx) and injected subcutaneously into rabbits followed by several booster injections.

Gel Filtration—HEK293 cells were lysed with buffer B and centrifuged at 15,000 rpm for 30 min. The resulting supernatant (200 μl) was applied on a column (1 × 30 cm) of Superdex 200 HR (Amersham Biosciences) that had been equilibrated with buffer B. Fractions (each 400 μl) were collected, and the aliquots were subjected to SDS-PAGE followed by Western blotting with anti-Tom1 and anti-Tollip antibodies.

Gel Filtration—HEK293 cells were lysed with buffer B and centrifuged at 15,000 rpm for 30 min. The resulting supernatant (200 μl) was applied on a column (1 × 30 cm) of Superdex 200 HR (Amersham Biosciences) that had been equilibrated with buffer B. Fractions (each 400 μl) were collected, and the aliquots were subjected to SDS-PAGE followed by Western blotting with anti-Tom1 and anti-Tollip antibodies.

Alternatively, the molecular mass was estimated by gel filtration—HPLC (Amersham Biosciences). To prepare anti-Tom1 and anti-Tollip antibodies, the thus purified Tom1-(180–492) and Tollip, respectively, were emulsified with Titer Max Gold (CytRx) and injected subcutaneously into rabbits followed by several booster injections.

Gel Filtration—HEK293 cells were lysed with buffer B and centrifuged at 15,000 rpm for 30 min. The resulting supernatant (200 μl) was applied on a column (1 × 30 cm) of Superdex 200 HR (Amersham Biosciences) that had been equilibrated with buffer B. Fractions (each 400 μl) were collected, and the aliquots were subjected to SDS-PAGE followed by Western blotting with anti-Tom1 and anti-Tollip antibodies.

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Fig. 1. Tom1 binds with Tollip. A, the extract of HEK293T cells that had expressed Flag-tagged Tom1, T7-tagged Tollip, or both was subjected to immunoprecipitation (IP) with anti-Flag tag antibody and to immunoblotting (IB) with anti-T7 tag or anti-Flag tag antibody. B, the extract of HEK293T cells was subjected to immunoprecipitation (IP) with anti-Tom1 antibody or control immunoglobulin G (IgG) and to immunoblotting (IB) with anti-T7 tag or anti-Tollip antibody. C, the extract of HEK293 cells was subjected to gel filtration on a Superdex 200 HR column (1 × 30 cm) in buffer B. Each fraction (400 μl) was analyzed by SDS-PAGE and immunoblotting (IB) with anti-Tom1 or anti-Tollip antibody. The peak fraction of Tom1 coincided with that of Tollip, and this peak is indicated by an open arrowhead. D, recombinant Tom1 (2 μg) was subjected to a GST pull-down assay after adding GST-Tollip fusion protein (left panel) and to immunoblotting (IB) with anti-Tom1 antibody (right panel). Left panel, SDS-PAGE of GST-Tollip fusion protein and immunoblotting (IB) with anti-GST antibody.

RESULTS

Identification of Tollip and Ubiquitin as Tom1-binding Proteins—By screening a yeast two-hybrid NIH3T3 MATCHMAKER cDNA library with the pGBKTT7-Tom1(1–310) plasmid, we obtained 27 positive clones from 1.4 × 105 independent ones. After sequencing, we found that they encode 13 different proteins. One of the positive clones encodes Tollip, which has been identified as an intermediate in interleukin-1 signaling (29). Tollip has a CUE domain, which has recently been found to be a ubiquitin-binding motif (31, 32), and has been demonstrated to bind with ubiquitin (31). Three other positive clones encode two ubiquitin-ribosomal protein fusion proteins and a polyubiquitin. Thus, our yeast two-hybrid screening revealed that Tollip and ubiquitin are candidates for Tom1-binding proteins.

Tom1 Interacts with Tollip, Ubiquitin, and Clathrin in vivo and in vitro

Tom1-interacting proteins were further examined using a yeast two-hybrid system. The interacting proteins were isolated using a GST-Tollip fusion protein (Fig. 2A (right panel) and B (right panel), respectively). Then, co-expressed Flag-tagged truncated mutant and T7-tagged full-length Tom1 or Tollip in HEK293T cells. The extracts of transfected cells were then subjected to immunoprecipitation using anti-Flag tag antibody and then to Western blotting with anti-T7 tag antibody to detect interaction (Fig. 2A). Tom1 was found to bind with Tollip. Next, to determine whether endogenous Tom1 interacts with endogenous Tollip or both was subjected to immunoprecipitation (IP) with anti-Flag tag antibody and to immunoblotting (IB) with anti-T7 tag or anti-Flag tag antibody. B, the extract of HEK293T cells was subjected to immunoprecipitation (IP) with anti-Tom1 antibody. The peak fraction of Tom1 coincided with that of Tollip, and this peak is indicated by an open arrowhead. D, recombinant Tom1 (2 μg) was subjected to a GST pull-down assay after adding GST-Tollip fusion protein (left panel) and to immunoblotting (IB) with anti-Tom1 antibody (right panel). Left panel, SDS-PAGE of GST-Tollip fusion protein and immunoblotting (IB) with anti-GST antibody.

again found that endogenous Tom1 interacts with endogenous Tollip. We next carried out gel filtration analysis to elucidate the formation of a Tom1-Tollip complex inside the cell. The extract of HEK293 cells was applied on a Superdex 200 HR column, and each fraction was subjected to SDS-PAGE followed by Western blotting with anti-Tom1 and anti-Tollip antibodies (Fig. 1C). Both Tom1 and Tollip were eluted at almost the same fraction with a molecular mass of ~350 kDa. Because the molecular masses of Tom1 and Tollip are 60 and 30 kDa, respectively, the complex formed is thought to be a multimer composed of Tom1 and Tollip or a multimer that binds with other proteins. It should be noted that Tom1 also exists in a higher molecular mass complex that lacks Tollip. Because Tom1 forms a complex with Tollip inside the cell, we carried out an experiment to determine whether Tom1 interacts directly with Tollip. Recombinant Tom1 was subjected to a GST pull-down assay after adding GST-Tollip fusion protein (Fig. 1D). Subsequent Western blotting with anti-Tom1 antibody revealed that Tom1 interacts directly with Tollip. Taken together, these results suggest that Tom1 binds with Tollip to form a complex in vivo and in vitro.

Determination of Domains of Tom1 and Tollip Required for Their Interactions—Because Tom1 can bind directly with Tollip, we carried out an experiment to determine their domains that are indispensable for their interactions. We constructed various Flag-tagged truncated mutants of Tom1 and Tollip shown in Fig. 2 (A (right panel) and B (right panel), respectively), and co-expressed the respective Flag-tagged truncated mutant and T7-tagged full-length Tom1 or Tollip in HEK293T cells. The extracts of transfected cells were then subjected to immunoprecipitation using anti-Flag tag antibody and then to Western blotting with anti-T7 tag antibody to detect interaction (Fig. 2, left panel). As shown in Fig. 2A, Tollip was found to bind efficiently with full-length Tom1 and truncated mutants of Tom1, Tom1(1–310) and -(180–492), both containing a GAT domain, but failed to bind with Tom1-(1–180), -(311–492), and -(Δ181–310), indicating that the GAT domain of Tom1 is indispensable for its interaction with Tollip.
On the other hand, Tom1 was found to bind with full-length Tollip and C-terminally truncated mutants of Tollip, Tollip(1–151) and (1–54), but failed to interact with Tollip(55–274) (Fig. 2B), indicating that the N-terminal region of Tollip is required for its interaction with Tom1. These domain analyses provide definitive evidence of interaction between Tom1 and Tollip.

Tom1 Is a Novel Ubiquitin-binding Protein—Because the results of yeast two-hybrid screening suggested that there is interaction between Tom1 and ubiquitin, we next carried out an experiment to determine whether Tom1 can bind with a ubiquitin and/or polyubiquitin chain. We expressed Flag-tagged Tom1 in HEK293T cells, and the extracts of transfected cells were subjected to immunoprecipitation using anti-Flag tag antibody and then to SDS-PAGE and subsequent immunoblotting with anti-ubiquitin antibody. As shown in Fig. 3A, Tom1 binds to a high molecular weight band that co-immunoprecipitates with Flag-tagged Tom1. To confirm the identity of this band, we performed a second round of immunoprecipitation using anti-Tom1 antibody and then subjected the immunoprecipitates to immunoblotting with anti-ubiquitin antibody. As shown in Fig. 3B, the smear bands detected originate from polyubiquitinated proteins that accumulated intracellularly.
with anti-Flag antibody-immobilized beads, were treated/denatured with 1% SDS to allow them to dissociate into Tom1 and polyubiquitinated proteins. Denatured Tom1 was treated with 1% Nonidet P-40 in a 10-fold volume and again subjected to the second immunoprecipitation and then to Western blotting as described above (Fig. 3A, 2nd). Signals caused by the polyubiquitin chains were undetectable in the second immunoprecipitation, although the signal caused by Flag-tagged Tom1 was detectable. Thus, these results rule out the possibility of polyubiquitination of Tom1 under the conditions used. It should be noted that monoubiquitinated Tom1 (Ub-Tom1 in Fig. 3A) was detected by Western blotting with anti-Flag tag antibody in the first and second immunoprecipitations (Fig. 3A, right panel), indicating that Tom1 is monoubiquitinated.

To demonstrate the interaction of endogenous Tom1 with endogenous polyubiquitinated proteins, extracts of HEK293T cells were subjected to immunoprecipitation using anti-Tom1 antibody and then to Western blotting with anti-ubiquitin antibody to detect interaction (Fig. 3B). It was again found that endogenous Tom1 is capable of binding with endogenous polyubiquitinated proteins. These results suggest that Tom1 is a novel ubiquitin chain-binding protein.

**Determination of the Domain of Tom1 Required for Its Interaction with Polyubiquitinated Proteins**

To determine the domain of Tom1 required for its binding with polyubiquitinated proteins, we constructed various Flag-tagged truncated mutants of Tom1 shown in Fig. 4A and expressed them in HEK293T cells. The extracts of transfected cells were then subjected to immunoprecipitation using anti-Flag tag antibody and then to Western blotting with anti-ubiquitin antibody (Fig. 4B). Polyubiquitinated proteins were found to be bound most efficiently to a C-terminally truncated mutant, Tom1-(1–310), moderately to full-length Tom1, and weakly to an N-terminally truncated mutant with deletion of half of the VHS domain, Tom1-(60–492), whereas the mutants with deletions of the GAT domain and the VHS domain, Tom1-(1–225) and Tom1-(148–492), respectively, did not bind with polyubiquitinated proteins. In cases of the former two mutants as well as full-length Tom1, two sequential immunoprecipitations with anti-Flag tag antibody were carried out using the extracts of HEK293T cells that had expressed Flag-tagged Tom1 and its truncated mutants, Tom1-(1–310) and (60–492), as in Fig. 3A. The band that originated from immunoglobulin G (IgG) is indicated by an arrowhead.

**Fig. 4. Analysis of Tom1 domain required for its interaction with polyubiquitinated proteins.** A and B, the extract of HEK293T cells that had expressed Flag-tagged full-length Tom1 (Tom1 WT) or its truncated mutant (A) and had subsequently been treated with 50 μM MG132 for 6 h was subjected to immunoprecipitation ([IP](IP)) with anti-Flag tag antibody and to immunoblotting ([IB](IB)) with anti-ubiquitin ([anti-Ub](anti-Ub)) or anti-Flag tag antibody ([anti-Flag](anti-Flag)). The levels of abilities to bind to polyubiquitinated proteins ([poly-Ub](poly-Ub)) are indicated on the right side of the respective domain structures (A). C, two sequential immunoprecipitations with anti-Flag tag antibody-immobilized beads were carried out using the extracts of HEK293T cells that had expressed Flag-tagged full-length Tom1 and its truncated mutants, Tom1-(1–310) and (60–492), as in Fig. 3A. The band that originated from immunoglobulin G ([IgG](IgG)) is indicated by an arrowhead.

**Fig. 5. Both Tom1 and Tollip bind with polyubiquitin chains.** A, a mixture consisting of 2 μg of monoubiquitin (Ub1) and 1 μg of polyubiquitin chains composed of 2–4 ubiquitin molecules (Ub2, Ub3, Ub4) was subjected to a GST pull-down assay after adding GST-Tom1 or GST-Tollip fusion protein ([left panel](left panel)) and to immunoblotting ([IB](IB)) with anti-ubiquitin ([anti-Ub](anti-Ub)) antibody ([right panel](right panel)). Left panel, SDS-PAGE and Coomassie Brilliant Blue ([CBB](CBB)) staining. B, the extract of HEK293T cells that had expressed Flag-tagged Tollip, T7-tagged Tom1, or both was subjected to immunoprecipitation ([IP](IP)) with anti-Flag tag or anti-T7 tag antibody and to immunoblotting ([IB](IB)) with anti-ubiquitin ([anti-Ub](anti-Ub)), anti-Flag tag, or anti-T7 tag antibody. Note that the levels of expression of Tom1 or Tollip in single transfections and simultaneous transfection are almost the same.

Noted that monoubiquitinated Tom1 (Ub-Tom1 in Fig. 3A) was detected by Western blotting with anti-Flag tag antibody in the first and second immunoprecipitations (Fig. 3A, right panel), indicating that Tom1 is monoubiquitinated.

To demonstrate the interaction of endogenous Tom1 with endogenous polyubiquitinated proteins, extracts of HEK293T cells were subjected to immunoprecipitation using anti-Tom1 antibody and then to Western blotting with anti-ubiquitin antibody to detect interaction (Fig. 3B). It was again found that endogenous Tom1 is capable of binding with endogenous polyubiquitinated proteins. These results suggest that Tom1 is a novel ubiquitin chain-binding protein.

**Determination of the Domain of Tom1 Required for Its Interaction with Polyubiquitinated Proteins**—To determine the domain of Tom1 required for its binding with polyubiquitinated proteins, we constructed various Flag-tagged truncated mutants of Tom1 shown in Fig. 4A and expressed them in HEK293T cells. The extracts of transfected cells were then subjected to immunoprecipitation using anti-Flag tag antibody and then to Western blotting with anti-ubiquitin antibody to detect interaction (Fig. 3B). It was again found that endogenous Tom1 is capable of binding with endogenous polyubiquitinated proteins. These results suggest that Tom1 is a novel ubiquitin chain-binding protein.
Flag antibody-immobilized beads were carried out as described in Fig. 3A (Fig. 4C). It was again found that signals caused by the polyubiquitin chains were undetectable in either case of the second immunoprecipitation. Thus, these results suggest that the region containing both of the GAT domain and the C-terminal part of the VHS domain is essential for the interaction of Tom1 with polyubiquitinated proteins. Because this region lacks canonical ubiquitin-binding motifs such as UIM and ubiquitin-associated domain (33), the GAT domain-containing region is thought to have a novel ubiquitin-binding motif. As shown in Fig. 2A, the GAT domain of Tom1 is required for the interaction of Tom1 with Tollip. On closer inspection, however, it was found that both the GAT domain and the C-terminal part of the VHS domain are required for the interaction of Tom1 with polyubiquitinated proteins, suggesting that the binding domains of Tom1 for Tollip and for the ubiquitin chain overlap but are not identical.

Both Tom1 and Tollip Interact with Polyubiquitin Chains—
As stated above, we found that Tom1 interacts with Tollip and with intraacellular polyubiquitinated proteins. It has recently been reported that Tollip directly binds with ubiquitin through its CUE domain (31). First, to determine whether there is direct interaction between Tom1 and the ubiquitin chain, a mixture consisting of monoubiquitin and polyubiquitin chains composed of 2–4 ubiquitin molecules was subjected to a GST pull-down assay after adding GST-Tom1 or GST-Tollip fusion protein (Fig. 5A, right panel). It was found that Tom1, as well as Tollip, binds directly with tetraubiquitin most efficiently in comparison with the cases of monoubiquitin and smaller ubiquitin chains. This finding indicates that there are the direct interactions of Tom1 and Tollip with ubiquitin chains. Because both Tom1 and Tollip have capacities to bind the ubiquitin chain, we next compared the ubiquitin binding ability of a complex composed of Tom1 and Tollip with that of Tom1 or Tollip (Fig. 5B). We expressed T7-tagged Tom1 only, Flag-tagged Tollip only, or both of them in HEK293T cells. The extracts of transfected cells were then subjected to immunoprecipitation using anti-Flag tag and anti-T7 tag antibodies and then to Western blotting with anti-ubiquitin, anti-Flag tag, and anti-T7 tag antibodies to detect the levels of ubiquitin binding, Tollip expression, and Tom1 expression, respectively. The level of polyubiquitinated proteins detected in the case of co-expression of Tom1 and Tollip was found to be much higher than those in the cases of single expressions of Tom1 and Tollip under the conditions in which the expression levels of Tom1 and Tollip alone were almost the same as levels of simultaneous expressions. From the results shown in Figs. 1 and 2, it is reasonable to assume that a complex of Tom1 and Tollip is formed in this co-expression experiment. Thus, these results strongly suggest that the Tom1-Tollip complex is capable of binding with polyubiquitinated proteins. With regard to the binding domains of Tom1 for Tollip and for the ubiquitin chain, it should be noted that the ubiquitin-binding capacity of Tom1 is not suppressed by the presence of Tollip and vice versa, suggesting that both Tom1 and Tollip in the complex can bind with the ubiquitin chains simultaneously. Alternatively, it is possible that complex formation between Tom1 and Tollip allosterically enhances the ability of one or the other component to bind with the ubiquitin chains.

Identification of Clathrin as a Tom1-binding Protein—
C-terminally truncated Tom1, Tom1-(1–310), was used as bait for the yeast two-hybrid screening in this study. To obtain more information on proteins that are able to interact with full-length Tom1, Tom1-(1–492), we carried out an experiment using the peptide mass fingerprinting method; Flag-tagged Tom1 was expressed in HEK293T cells, and the extracts of transfected cells were then subjected to immunoprecipitation using anti-Flag tag antibody. Proteins in the immunoprecipitates were separated by SDS-PAGE, and the separated proteins were excised and digested in gel with trypsin. The peptides thus produced were subjected to mass fingerprinting. By a search of a mass spectrometry data base, one of the proteins that bound to Tom1 was identified as the heavy chain of clathrin. This finding strongly suggests that clathrin is a Tom1-binding protein. It is well known that various proteins interact with clathrin through their canonical clathrin boxes with the consensus sequence pLxMxxL (p, polar amino acid; M, hydrophobic amino acid) (9). A search for a clathrin box in the amino acid sequence of Tom1 revealed that Tom1 has a canonical clathrin box at sequence 321–326, Asp-Leu-Ile-Asp-Met-Gly, as shown in Fig. 6A. To determine whether Tom1 is capable of interacting with clathrin through its clathrin box, Flag-tagged Tom1 or its mutant lacking clathrin box, Tom1-(321–325), was expressed in HEK293T cells, and extracts of transfected cells were subjected to immunoprecipitation using anti-Flag tag antibody and then to Western blotting with anti-clathrin antibody to detect interaction (Fig. 6B). Tom1 was found to interact with endogenous clathrin, whereas the mutant with deletion of the clathrin box scarcely bound to clathrin, indicating clathrin box-dependent interaction of Tom1 with clathrin. To obtain evidence of interaction between endog-
enous Tom1 and endogenous clathrin, HEK293T cells were lysed, and the resultant extract was subjected to immunoprecipitation using anti-Tom1 antibody and then to Western blotting with anti-clathrin antibody to detect interaction (Fig. 6C). It was again found that endogenous Tom1 interacts with endogenous clathrin.

**Intracellular Localization of GFP-Tom1**—To determine whether the intracellular localization of Tom1 protein is dependent on its clathrin box, we expressed GFP-Tom1 fusion protein and GFP-Tom1-Δ321–325 fusion protein in NIH3T3 cells. The cells were fixed and stained with DAPI, and fluorescence as a result of GFP and DAPI was observed under a fluorescence microscope (Fig. 7). It was found that GFP-Tom1 fusion protein was localized predominantly in the cytoplasm (Fig. 7B), whereas GFP-Tom1-Δ321–325 fusion protein had a diffuse localization throughout the cell (Fig. 7C), as was the case with GFP (Fig. 7A), indicating that the cytoplasmic localization of Tom1 is dependent on its clathrin box. Based on the finding that Tom1 interacts with clathrin in a clathrin box-dependent manner together with the results of this microscopic observation, we propose that Tom1 is localized in the cytoplasm through its binding to clathrin, which is localized and functions in the cytoplasm (8).

**DISCUSSION**

In this study, we found that Tom1, a VHS domain-containing protein, binds with Tollip, polyubiquitin chain, and clathrin. Tom1 associates with Tollip to form a high molecular mass complex. The GAT domain of Tom1 and the N-terminal region of Tollip are required for their interactions. Tom1 was found to be a novel ubiquitin-binding protein. This protein prefers polyubiquitin chain to monoubiquitin, and the region containing the GAT domain and the C-terminal part of the VHS domain is indispensable for ubiquitin binding. In addition, Tom1 has a canonical clathrin box and this box is indispensable for clathrin binding. Based on the above findings, we propose a model (Fig. 8) in which a complex of Tom1 and Tollip, both of which are capable of binding with polyubiquitin chains, recruits polyubiquitinated proteins to clathrin.

Tollip, a binding partner of Tom1 (Fig. 1), was initially isolated as a mediator of interleukin-1 signaling (29). In the interleukin-1 signaling pathway, binding of interleukin-1 to its receptor results in the assembly of a membrane-proximal signaling complex that consists of the receptor, the receptor accessory protein, an adaptor protein (MyD88), an interleukin-1 receptor-associated-kinase (IRAK), and Tollip. It has been reported that Tollip forms a complex with IRAK in resting cells, whereas the Tollip-IRAK complex is disrupted after interleukin-1 treatment (29). Gel filtration analysis in this study showed that Tollip forms a high molecular mass complex with Tom1 (Fig. 1C). A stable cell line of HEK293 cells that constitutively expresses the interleukin-1 receptor was established. These cells were stimulated with interleukin-1, but the patterns of Tom1 and Tollip in gel filtration were found to be the same as those shown in Fig. 1C and remained unchanged after stimulation with interleukin-1 (data not shown). In addition, Western blotting of gel filtration fractions with anti-IRAK antibody together with anti-Tollip and anti-Tom1 antibodies revealed that the elution pattern of IRAK was not coincident with those of Tollip and Tom1 (data not shown). The discrepancy between our results and the results of a previous study (29) might be caused by the difference in the cell lines used. Alternatively, a small population of Tollip could form a complex with IRAK. In any case, at least under the conditions used in this study, it can be concluded that a large population of Tollip binds with Tom1 irrespective of interleukin-1 stimulation.

Tom1 has a GAT domain of a smaller size than those in GGA proteins (15). The GAT domain of Tom1 was found to be indispensable for its interaction with Tollip (Fig. 2A), whereas the large-size GAT domains in GGA proteins are required for their interaction with ARF (26–28). The crystal structure of the GAT domain of GGA1 protein was found to be a three-helix bundle with a long N-terminal helical extension (26–28). The amino acid sequence of the bundle in GGA1 is conserved in Tom1, whereas that of the extension in GGA1 is not conserved in Tom1. The N-terminal helical extension in GGA1 was also found to be indispensable for its interaction with ARF, but the function of the bundle has not been determined. A comparison of predicted secondary structures suggests that the small GAT domain of Tom1 is folded into a three-helix bundle similar to
that in GGA1 (data not shown). Because this domain of Tom1 is essential for its interaction with Tollip, it is reasonable to assume that the corresponding region of GGA1 is required for the interaction of GGA1 with its putative binding partner.

Tom1, as well as Tollip, was found to interact with polyubiquitin chain (Fig. 3). The region consisting of the GAT domain and the C-terminal part of the VHS domain is indispensable for the interaction of Tom1 with polyubiquitin chains (Fig. 4). A Tom1 mutant with deletion of the N-terminal part of the VHS domain has the ability to bind polyubiquitinated proteins, whereas that with deletion of the C-terminal part of the GAT domain cannot bind polyubiquitinated proteins (Fig. 4). It is therefore thought that the GAT domain plays a more essential role in ubiquitin binding than the VHS domain does. The above binding region lacks canonical ubiquitin binding motifs such as UIM (5) and ubiquitin-associated domain (33). Although the critical sequence in the above region required for ubiquitin binding has not yet been determined, Tom1 can be considered to be a novel type of ubiquitin-binding protein. With regard to the specificity of ubiquitin binding, it should be noted that Tom1 has a stronger ability to bind tetraubiquitin than smaller ubiquitin chains, including monoubiquitin (Fig. 5A).

Because the polyubiquitin chain used in this study is formed via a covalent bond between the Lys-48 residue of the original ubiquitin molecule and the Gly-76 residue in the C terminus of the subsequent ubiquitin molecule (34), it can be concluded that Tom1 has the ability to bind the Lys-48-linked polyubiquitin chain, although the Lys-48-linked polyubiquitin chain is thought to function as a signal for proteasome-mediated protein degradation (34).

Clathrin is a major component of the vesicle coat (8), and various proteins have been reported to interact with clathrin in clathrin-mediated vesicular trafficking (9). Tom1 was found to be a new clathrin-binding protein and to have a canonical clathrin box (Fig. 6B) and also for its cytoplasmic localization (Fig. 7). Among VHS domain-containing proteins, STAM and Hrs bind to form a complex, and this complex is able to bind with ubiquitin through their UIMs (13, 14). Hrs also binds to clathrin through its canonical clathrin box (35). These properties of STAM and Hrs, i.e., complex formation, ubiquitin binding, and clathrin binding, appear to be the same as those of Tom1 and Tollip found in this study. This similarity, together with the fact that the STAM-Hrs complex functions in clathrin-mediated endosomal protein sorting (13, 14), leads us to propose an idea that the Tom1-Tollip complex participates in clathrin-mediated events.

It has been reported that the STAM-Hrs complex binds with Eps15 to form a ternary complex (14). Eps15, a member of the family of EH (which stands for Eps15 homology) domain-containing proteins (36), is a component of clathrin-coated pits (37), and the EH domain of Eps15 recognizes the motif NPF (Asn-Pro-Phe) (36, 38). Tom1 has the sequence NPF at its N terminus, but it has been reported that the NPF sequence of Tom1 fails to bind the EH domain (22). Thus, the function of the VHS domain containing the NPF sequence in Tom1 remains unclear. On the other hand, Tom1 was found to be monoubiquitinated (Fig. 3) as is the case with Hrs (10). Monoubiquitin linked to Tom1 may function as a signal for recruitment of other ubiquitin-binding proteins, including UIM-containing proteins (4–6). This monoubiquitination of Tom1, together with the fact that Tom1 and Tollip form a high molecular mass complex, suggests that the Tom1-Tollip complex binds with other proteins, as is also likely in the case of the STAM-Hrs-Eps15 ternary complex. Structural and cytological analyses of this large complex formed with Tom1 and Tollip are needed to understand the function of Tom1 in clathrin-mediated events.

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