Ubiquitin Interacts with the Tollip C2 and CUE Domains and Inhibits Binding of Tollip to Phosphoinositides*

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Background: Tollip participates in endosomal membrane trafficking and innate immune pathways.

Results: Ubiquitin inhibits binding of Tollip to phosphatidylinositol 3-phosphate by association to the Tollip C2 and CUE domains and dissociates Tollip CUE domain dimers.

Conclusion: Ubiquitin negatively modulates membrane association of Tollip.

Significance: Ubiquitin dual binding can partition Tollip into membrane-bound and cytosolic intracellular pools.

A large number of cellular signaling processes are directed through internalization, via endocytosis, of polyubiquitinated cargo proteins. Tollip is an adaptor protein that facilitates endosomal cargo sorting for lysosomal degradation. Tollip preferentially binds phosphatidylinositol 3-phosphate (PtdIns(3)P) via its C2 domain, an association that may be required for endosomal membrane targeting. Here, we show that Tollip binds ubiquitin through its C2 and CUE domains and that its association with the C2 domain inhibits PtdIns(3)P binding. NMR analysis demonstrates that the C2 and CUE domains bind to overlapping sites on ubiquitin, suggesting that two ubiquitin molecules associate with Tollip simultaneously. Hydrodynamic studies reveal that ubiquitin forms heterodimers with the CUE domain, indicating that the association disrupts the dimeric state of the CUE domain. We propose that, in the absence of polyubiquitinated cargo, the dual binding of ubiquitin partitions Tollip into membrane-bound and membrane-free states, a function that contributes to the engagement of Tollip in both membrane trafficking and cytosolic pathways.

Post-translational modification of proteins, including phosphorylation, glycosylation, lipidation, acetylation, and methylation, represents key mechanisms that cells use to respond to environmental changes and to modulate protein function. An additional layer of protein regulation is the covalent attachment of ubiquitin to proteins, a process called ubiquitination. Although ubiquitination commonly signals for protein degradation by the proteasome machinery, it can also lead to other nondegradative pathways, including DNA repair, cellular trafficking, chromatin remodeling, and immune responses (1).

Ubiquitin is a 76-amino acid globular protein that can be attached as a single unit (monoubiquitination), or attached as a polyubiquitin chain. Monoubiquitination leads to the conjugation of a single ubiquitin molecule onto one or more lysine residues on the target proteins’ surface. Polymerization of ubiquitin occurs by conjugation of any of the ε-amino groups on the seven surface lysine residues or the N-terminal methionine to the C-terminal glycine (2). In addition, ubiquitin is recognized by ubiquitin-binding domains (also known as ubiquitin receptors), which translate ubiquitinated target signals into cell signaling cascades. Over 20 distinct ubiquitin-binding domains associate noncovalently with ubiquitin moieties with affinities in the micromolar range (1), which can be explained by the fact that the intracellular ubiquitin concentration can be as high as 85 μM (3). The majority of the ubiquitin-binding domains bind to ubiquitin through a hydrophobic patch in which the Leu8, Ile44, and Val70 residues of ubiquitin play critical roles (4). Ubiquitin-binding domains are structurally classified in α-helical, zinc fingers, pleckstrin homology folds, and ubiquitin-conjugating-like domains (1). The coupling of ubiquitin conjugation to endosomal reticulum degradation (CUE), GGA and Tom1 (GAT), and Vps27/Hrs/Stam (VHS) domains belong to the helical subfamily of ubiquitin-binding domains (1). Despite the fact that the ubiquitin backbone is rigid (with the exception of its C terminus), association of ubiquitin-binding domains leads to conformational changes in ubiquitin (5). Ubiquitin-binding domains display more selectivity and bind more tightly to certain types of polyubiquitinated chains, in which these polymers can adopt packed or extended conformations depending on what lysine residues link ubiquitin chains (reviewed in Ref. 1). Several ubiquitin-binding domains have been shown to mediate intra-monoubiquitination, leading to a ubiquitin-binding domain-containing protein conformation that exhibits altered function and localization and likely alters its interactions with ubiquitinated cargo during endosomal trafficking (reviewed in Ref. 6).

To control cellular processes, cells dampen signaling through a broad range of mechanisms including cargo internalization. The mechanism of cargo removal includes its internalization by endocytosis, followed by ubiquitin-mediated delivery to early endosomes, where cargo sorting takes place (7). Cargo is further sorted into intralumenal vesicles of late endosomes/multivesicular bodies. Alternatively, some cargo is retrieved...
from this pathway. Once all the retrieved molecules have been removed, late endosomes/multivesicular bodies fuse with lysosomes leading to cargo degradation in the vesicular lumen (8). Adaptor proteins regulate protein sorting by interacting with different macromolecules at the surface of endosomal membranes. The function of many adaptor proteins is associated with their ubiquitin-binding domains, ensuring the precise sorting of ubiquitinated cargo proteins inside the intraluminal endosomal vesicles. One of these adaptor proteins is Tollip, which is involved in protein sorting by association with Tom1, ubiquitin, and clathrin (9). Tollip is primarily localized on early endosomes where it is required for degradation of ubiquitin-conjugated cargo (10), including sorting of the interleukin-1 receptor (IL-1R) (11) and the transforming growth factor receptor-β type I receptor (12) for their degradation. Tollip also controls innate immune responses by regulating IL-1R-associated kinase-1 (IRAK-1)5 function in both the Toll-like receptor and the IL-1R signaling pathways (10, 13, 14). By binding to IRAK proteins, Tollip indirectly regulates the function of transcription factors, such as nuclear factor (NF)-κB, to influence the expression of innate immune-related genes, such as those that codify for cytokines and interferons (15). Moreover, Tollip has been shown to be sumoylated and mediates IL-1R sumoylation (16), placing Tollip as a modulator of nuclear and cytoplasmic functions. In addition to the Tollip CUE domain, we identify the Tollip C2 domain as a novel ubiquitin-binding domain. By binding to Tollip disrupts Tollip-phosphoinositide interactions. In addition to the Tollip CUE domain, we identify the Tollip C2 domain as a novel ubiquitin-binding domain. By comparison, Tollip C2 and CUE domains bind ubiquitin in partially overlapping sites with moderate affinity, with the Tollip C2 domain showing an extensive contact surface, whereas the corresponding site for the CUE domain is mapped on an exposed hydrophobic patch around Ile-44. Remarkably, we demonstrate that the dimeric Tollip CUE domain dissociates when bound to ubiquitin. Based on our findings, we propose that ubiquitin binds to two independent sites in Tollip, an association that promotes dissociation of the Tollip CUE domain dimers, and direct inhibition of PtdIns(3)P ligation, leading to membrane release of adaptor protein complexes in the absence of polyubiquitinated cargo.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—1,2-Dioly1-sn-glycero-3-phosphocholine (PtdCho), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PtdEtn), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns) were from Avanti Polar Lipids; PtdIns(3)P and PtdIns(4,5)P2 were from Echelon Biosciences. All other chemicals were analytical reagent grade.

**Cloning, Expression, and Purification of Tollip and Ubiquitin Constructs**—The cloning, expression, and purification of human Tollip and the isolated C2 (residues 54–182) and CUE (residues 223–278) domains as GST fusions and untagged proteins were carried out as previously reported (18, 21). Purified His-tagged rat PKCa C2 domain and a rat cDNA that codifies for PKCβ II C2 domain, cloned into the pGEX4T1 vector, were kindly provided by Dr. Robert Stahelin (University of Notre Dame). The cDNA of human ubiquitin, cloned into the pET24d vector, was a generous gift from Dr. Julie Forman-Kay (University of Toronto). Site-directed mutagenesis was performed using QuikChange (Stratagene). Ubiquitin constructs were expressed in *Escherichia coli* Rosetta strain (Stratagene). Ubiquitin was generated in Luria-Bertani media, whereas the 15N-labeled form was produced in minimal media supplemented with 15NH4Cl (Cambridge Isotope Laboratory Inc.) as the source of nitrogen. Induction of the His-tagged fusion ubiquitin was performed by the addition of 1 mm isopropyl 1-thio-β-D-galactopyranoside to the bacterial cell culture at an OD of ~0.8 followed by a 2-h incubation at 25 °C. Cell pellets were suspended in ice-cold equilibrium buffer containing 50 mm sodium phosphate (pH 8), 300 mm NaCl, 0.1 mg/ml of lysozyme, 1 mm β-mercaptoethanol, 1 mM leupeptin, 1 mM pepstatin, 8.6 μg/ml of 1-β-hexosaminidase, 4.3 μg/ml of 1-chloro-3- tosylamido-7-amino-2-heptanone, 0.15 μg/ml of aprotinin, 0.1% Triton X-100, 0.1% Tween 20, and 0.1% Nonidet P-40. Suspension was further processed by sonication and centrifugation and the resulting supernatant was incubated with Talon metal affinity resin (Clontech). In some cases, fusion proteins were eluted off the beads by the addition of a buffer containing 50 mm sodium phosphate (pH 8), 1 mm NaCl, and 200 mm imidazole. For untagged protein purifications, His tag was removed by incubation of the fusion protein with tobacco etch virus protease in buffer containing 50 mm sodium phosphate (pH 8), 100 mm NaCl, 10% glycerol, 1 mm DTT, and 0.5 mm EDTA at room temperature for 3 days. Proteins were recovered in a buffer containing 50 mm sodium phosphate (pH 8), 300 mm NaCl, and 1 mm DTT, and concentrated using a 2-kDa cut-off concentrator device (Millipore) and further purified by an ÄKTA FPLC system using a Superdex 30 column (GE Healthcare), previously equilibrated with 50 mm Tris-HCl (pH 8), 200 mm NaCl.

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5 The abbreviations used are: IRAK, IL-1R-associated kinase; CD, circular dichroism; HSQC, heteronuclear single quantum coherence; IL-1R, interleukin-1 receptor; NMR, nuclear magnetic resonance; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdSer, phosphatidylserine; SPR, surface plasmon resonance; TBD, Tom1-binding domain; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate.
and 1 mM DTT. Protein concentrations were calculated using the BCA method. Purity of proteins was over 95% as judged by SDS-PAGE and MALDI-TOF mass spectrometry analyses and by their identity using N-terminal sequencing (Tufts University).

**Liposome Preparation**—Stocks of PtdIns(3)P, PtdCho, and PtdEth, were prepared in organic solvents using chloroform: methanol:water (65:35:8). PtdIns(3)P liposomes were prepared by weight ratio of 1:1 of PtdCho:PtdEth and 10% PtdIns(3)P. Control liposomes contained 1:1 of PtdCho:PtdEth. Lipid films were generated in a desiccator overnight and hydrated in 20 mM Tris-HCl (pH 6.8) and 100 mM NaCl to 1 mg/ml at 60 °C for 1 h. Liposomes were sonicated, pelleted, and suspended at 2.5 mg/ml in the same buffer and then subjected to extrusion at 60 °C using 400-nm membranes and immediately used for SPR experiments.

**Lipid-Protein Overlay Assay**—Lipid strips were prepared by spotting 1 μl of the indicated amount of PtdIns(3)P dissolved in chloroform/methanol/water (65:35:8) onto Hybond-C extra membranes (GE Healthcare). Membrane strips containing the immobilized PtdIns(3)P were blocked with 3% (w/v) fatty acid-free BSA (Sigma) in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. In some cases, GST fusion proteins were preincubated with 2–4-fold excess molar ratios of ubiquitin. Then, membranes were incubated with ubiquitin-free or protein/ubiquitin mixtures in the same buffer overnight at 4 °C. Following four washes with the same buffer, bound proteins were probed with rabbit anti-GST antibody (Santa Cruz Biotechnology) and donkey anti-rabbit horseradish peroxidase antibody (GE Healthcare). Protein binding was detected using Supersignal West Pico chemiluminescent reagent (Pierce). In the case of PtdSer binding studies of the His-PKCa C2 domain, in the absence and presence of ubiquitin, bound protein was detected using rabbit anti-His antibody (Rockland Inc.).

**SPR Analysis**—The kinetics and affinity of protein-protein and protein-lipid interactions were determined using a BIAcore X100 instrument. For ubiquitin interactions, 200 nM His-tagged ubiquitin (ligand) was immobilized using a NiCl₂-activated NTA sensor chip (GE Healthcare) surface and used as a ligand, whereas untagged Tollip CUE or C2 domains, or GST-Tollip were flown as analyte. The first flow cell of the sensor chip was used as a control surface (no protein), whereas the second flow cell was employed as the active surface. His-tagged ubiquitin, in a buffer containing 10 mM HEPES (pH 8.3), 150 mM NaCl, 0.005% Tween 20, 50 μM EDTA, and 1 mM NaN₃, was immobilized on the surface of one flow cell of the sensor chip. A range of concentrations of protein analytes prepared in the same buffer was injected on both flow cell surfaces at a flow rate of 30 μl/min. Association and dissociation times for each protein injection were set at 120 and 600 s, respectively. The remaining bound protein was washed away by the injection of 30 μl of 10 mM HEPES (pH 8.3), 150 mM NaCl, 0.005% Tween 20, 350 μM EDTA, and 1 mM NaNO₃. Tollip-PtdIns(3)P interactions were followed using PtdIns(3)P-free and -enriched liposomes as ligands and GST-Tollip as an analyte, similar to what we previously described (18). For the competition experiments, GST-Tollip (900 nM) was preincubated with increasing molar concentrations of untagged ubiquitin for 1 h at room temperature. Free and ubiquitin-bound GST-Tollip was then flown over
PtdIns(3)P-free and PtdIns(3)P-enriched liposomes, which were immobilized on the L1 sensor chip surface. To estimate the kinetic parameters, sensorgrams best fit to the two-state (conformational change) and bivalent analyte binding models using BIAcore X100 evaluation software (version 2.0). The values were less than 10% of the maximal binding response ($R_{\text{max}}$) for each data set.

**NMR Spectroscopy**—NMR data were acquired at 25 °C using a Bruker Avance III 600 MHz spectrometer (Virginia Tech) equipped with an inverse TBI probe with $z$ axis pulsed-field gradients. Two-dimensional $^1$H,$^{15}$N-heteronuclear single quantum coherence (HSQC) experiments were used to track backbone amide resonance changes in $^{15}$N-labeled proteins due to interaction with the unlabeled protein partner. For ubiquitin-Tollip C2 domain interactions, 30 μM ubiquitin was prepared in 20 mM $d_{11}$-Tris-HCl (pH 7.5), 350 mM NaCl, 1 mM NaN$_3$, and 1 mM $d_{18}$-DTT and unlabeled Tollip C2 domain was added at 8-fold excess to visualize resonance perturbations. In the case of ubiquitin-Tollip CUE domain interactions, 50 μM $^{15}$N-Tollip CUE domain was prepared in 20 mM phosphate buffer (pH 7) and unlabeled ubiquitin was added at 2-fold excess. Reciprocally, 50 μM $^{15}$N-ubiquitin was prepared in the same buffer and unlabeled Tollip CUE domain was added at 2-fold excess. Spectra were processed with NMRPipe (22) and analyzed using nmrDraw (23). Chemical shift perturbations were calculated according to the following formula (24).

$$\Delta \delta_{i}^{(1H,^{15}N)} = [(\Delta \delta_{i}^{1H})^2 + (\Delta \delta_{i}^{15N})^2]^{0.5} \quad \text{(Eq. 1)}$$

**Circular Dichroism Spectroscopy**—Far-UV (190–240 nm) circular dichroism (CD) experiments were performed on a JASCO J-815 spectropolarimeter equipped with a Jasco PFD-425 S temperature control unit. Five accumulated untagged protein (10 μM) CD spectra were recorded in 5 mM Tris-HCl (pH 7.0) and 100 mM KF at 25 °C using a 1-mm path-length quartz cell, a bandwidth of 4 nm, a response time of 1 s, and a scan rate of 20 nm/min. Buffer backgrounds were subtracted from the protein spectra.

**Analytical Gel Filtration**—Various concentrations of free protein and complex were incubated in 50 mM Tris-HCl (pH 8), 200 mM NaCl, and 1 mM DTT for 30 min at 25 °C. Protein samples (1.5 ml) were applied to a Superdex 75 column (GE Healthcare), equilibrated with the same buffer, and run at 1 ml/min at 25 °C. Protein samples were collected and analyzed by 10% SDS-PAGE and Coomassie Blue staining. The column was calibrated with protein standards (blue dextran, BSA, ovalbumin, chymotrypsinogen A, myoglobin, ribonuclease A, and aprotinin). Stokes radii of the proteins were determined by logarithmic interpolation.

**Analytical Ultracentrifugation**—Sedimentation velocity experiments with Tollip CUE (605 μM), ubiquitin (900 μM), and a 1:1 molar ratio of the Tollip CUE-ubiquitin complex (350 μM each) were carried out using a Beckman Optima XL-I ultracentrifuge equipped with a 50Ti 8-hole rotor and using a 12-mm path length cell (Biophysics Core, University of Colorado, Denver, CO). Absorbance profiles at 273 nm were collected every 30 s at 45,000 rpm and 25 °C. Sedimentation coefficient continuous c(S) distributions were determined using SEDFIT software (25). The resulting apparent sedimentation coefficients were corrected to standard conditions as reported (26). The calculated molecular weight of the protein was estimated using the Siegel-Monty analysis (27).

### TABLE 1

| Complex            | $k_{a1}$ (M$^{-1}$ s$^{-1}$) | $k_{d1}$ (s$^{-1}$) | $k_{a2}$ (s$^{-1}$) | $k_{d2}$ (s$^{-1}$) | $K_D$ (M) | Fit ($\chi^2$) |
|--------------------|-----------------------------|---------------------|---------------------|---------------------|------------|----------------|
| Tollip-PtdIns(3)P  | 8.8 ± 0.3 x 10$^2$          | 7.4 ± 1.3 x 10$^{-4}$ | 1.4 ± 0.1 x 10$^{-3}$ | 3.9 ± 1.5 x 10$^{-4}$ | 4.60 x 10$^{-8}$ | 0.28          |
| Tollip C2-ubiquitin| 8.3 ± 0.1 x 10$^3$          | 3.8 ± 0.1 x 10$^{-2}$ | 8.4 ± 0.1 x 10$^{-4}$ | 9.6 ± 0.1 x 10$^{-3}$ | 1.08 x 10$^{-5}$ | 0.83          |
| Tollip CUE-ubiquitin| 3.0 ± 0.3 x 10$^4$          | 2.4 ± 0.1 x 10$^{-1}$ | 2.1 ± 0.1 x 10$^{-3}$ | 1.2 ± 0.1 x 10$^{-4}$ | 1.35 x 10$^{-6}$ | 3.06          |

### Following a two-state conformational change model

### Following a bivalent analyte model
RESULTS

Ubiquitin Inhibits the PtdIns(3)P Binding of Tollip—
Tollip localizes to endosomal membranes and the subcellular localization of the protein is likely associated with its ability to preferentially bind PtdIns(3)P through its central C2 domain (18). Because Tollip binds ubiquitin through its CUE domain (19), we then asked whether ubiquitin regulates phosphoinositide recognition. Whereas Tollip binds PtdIns(3)P and ubiquitin does not, preincubation of Tollip with ubiquitin led to inhibition of PtdIns(3)P Tollip binding in a dose-dependent manner (Fig. 1A). Likewise, ubiquitin also inhibited PtdIns(4,5)P_2 binding of Tollip (Fig. 1B). Tollip reversibly bound PtdIns(3)P-containing liposomes with a dissociation constant (K_d) of 46 nM (Fig. 1C and Table 1). To quantify the effect of ubiquitin on Tollip-PtdIns(3)P association, we performed a competition experiment using SPR analysis. Preincubation of Tollip with

FIGURE 2. The Tollip C2 domain is an ubiquitin-binding domain. A, lipid-protein overlay assay of the indicated proteins with immobilized PtdIns(3)P. B, lipid-protein overlay assay of the Vam7p PX domain with immobilized PtdIns(3)P and PKCα and PKCβ II C2 domains with immobilized PtdSer in the absence and presence of ubiquitin. C, representative SPR sensogram for binding of the Tollip C2 domain to immobilized ubiquitin. Various concentrations of Tollip C2 were flown over His-tagged ubiquitin attached on an NTA sensor chip. D, identification of the ubiquitin residues involved in Tollip C2 domain recognition. 15N-Labeled ubiquitin was subjected to HSQC analysis in the absence (black) and presence (red) of the Tollip C2 domain. Perturbed ubiquitin resonances are boxed. E, histogram identifying ubiquitin critical residues for Tollip C2 domain recognition. The colored dashed lines represent significant changes, based on the magnitude of their associated chemical shifts changes: red (Δ_δ_{average} + 1.5 × S.D.) > orange (Δ_δ_{average} + 1 × S.D.) > yellow (Δ_δ_{average}). F, two different views of ubiquitin showing the residues involved in Tollip C2 domain binding and color-coded according to the scales defined in E.
ubiquitin reduced PtdIns(3)P association in a ubiquitin dose-dependent manner (Fig. 1D) with an estimated IC_{50} of 3.8 μM.

The Tollip C2 Domain Is an Ubiquitin-binding Domain—To make sure that the effects we observed in Fig. 1 were due to the ability of the Tollip CUE domain to bind to ubiquitin, we performed a control experiment using the Tollip C2 domain. To our surprise, ubiquitin inhibited PtdIns(3)P binding to the Tollip C2 domain (Fig. 2A). Binding of PtdSer of both PKCα and PKCβ II C2 domains was also reduced by ubiquitin, but this protein did not affect Vam7p PX domain PtdIns(3)P binding (Fig. 2B), suggesting a common ubiquitin-mediated mechanism of inhibition of lipid binding of C2 domains. Next, SPR was used to determine whether the Tollip C2 domain binds ubiquitin. His-tagged ubiquitin was immobilized on the surface of an NTA sensor chip, and the untagged Tollip C2 domain was employed as an analyte. When the Tollip C2 domain was flown over the sensor chip, binding to ubiquitin was observed in a concentration-dependent manner and exhibited rapid association and dissociation rates (Fig. 2C). The binding event best fit with the two-state conformational change model. The Tollip C2 domain bound ubiquitin with a K_D of 10.8 μM (Table 1), which was determined from the kinetic rate constants. This value is within the range of the affinities reported for other ubiquitin-binding domains (28). Binding of the Tollip C2 domain to ubiquitin exhibited a saturable binding trace that is indicative of specific binding. To map the C2 domain-binding site of Tollip in ubiquitin, we collected \(^{1}H,^{15}N\)-HSQC spectra of \(^{15}N\)-labeled ubiquitin. Changes in the position of the HSQC chemical shifts can be induced by ligand binding or by changes in structure and/or dynamics of the protein. Addition of unla-lebelled Tollip C2 domain to the \(^{15}N\)-labeled ubiquitin led to a large number of perturbations in chemical shifts in the HSQC spectrum of ubiquitin (Fig. 2D). Large chemical shift changes were observed in ubiquitin residues Asp-32 and Lys-33 located at the end of the first helix, residues Gly-47 and Gln-51 located around the fourth β-strand element, and Gln-62 and Leu-71 found around the fifth β-strand element (Fig. 2E). Minor perturbations were also detected in residues around Gly-47 and Leu-71 such as Lys-6, Gly-10, Lys-11, Leu-43, Ile-44, Leu-69, Val-70, and Leu-73, which form a relatively hydrophobic patch-binding site characteristic of protein interactions of ubiquitin (Fig. 2F). Thus, these results suggest that a large region of the surface of ubiquitin is engaged in Tollip C2 domain binding. To identify key Tollip C2-ubiquitin interacting residues, we performed alanine mutagenesis on ubiquitin residues, whose resonances were perturbed by the presence of the Tollip C2 domain, and evaluated the effect of these mutations using SPR detection. Mutations in residues located in and around the hydrophobic patch (Leu-43) and in residues located at the C-terminal flexible region of ubiquitin (i.e. Leu-71) reduced Tollip C2 domain affinity about 2–3-fold (Table 2). Mutations in Leu-71 neighbor residues, such as Lys-6 and Leu-73, did not affect Tollip C2 domain binding (Table 2). Identification of the ubiquitin-binding residues in the Tollip C2 domain using NMR spectroscopy was not possible because we were unable to obtain a stable Tollip C2 domain sample at the concentration required to assign its NMR resonances. Nonetheless, alanine mutation in a Tollip C2 domain basic residue important for PtdIns(3)P binding (Lys-162) reduced the affinity for ubiquitin 2-fold, but mutations in other PtdIns(3)P-binding residues (His-135, Arg-157) did not affect ubiquitin binding (Table 2). Using the predicted structure of the Tollip C2 domain (18), we found that the conserved residue Leu-142 is close to Lys-162. Alanine mutation in both Leu-142 and Lys-162 reduced the affinity for ubiquitin 6-fold, without altering the overall structure of the protein (Table 2 and data not shown). Taken together, these results suggest that PtdIns(3)P and ubiquitin-binding sites in the Tollip C2 domain partially overlap.

The Tollip C2 and CUE Domain Binding Sites in Ubiquitin Partially Overlap—The data indicated that both the Tollip CUE and C2 domains bind to ubiquitin, suggesting that ubiquitin binds at two sites on Tollip with distinct affinities. Alternatively, it is also possible that one molecule of ubiquitin binds both domains at different interfaces leaving Tollip in a closed conformation. To address this, we identified ubiquitin residues that associate with the Tollip CUE domain using HSQC chemical shift-based analysis. Ubiquitin bound the Tollip CUE domain and triggered a large number of chemical shift changes in the ubiquitin spectrum (Fig. 3A). Drastically perturbed ubiquitin residues include the N-terminal Leu-8 and Ile-13 residues, the surface hydrophobic protein-interacting residues Leu-43, Ile-44, and Gly-47, and the C-terminal residues Gln-62, Leu-67, His-68, Leu-69, and Val-70 (Fig. 3B). Most of these residues form a relatively hydrophobic binding site on the ubiquitin surface (Fig. 3C). Several ubiquitin resonances that are perturbed by the Tollip CUE domain, such as those corresponding to Ile-13, Leu-43, Ile-44, Gly-47, and Gln-62, to mention a few, were also perturbed by the presence of the Tollip C2 domain (Figs. 2 and 3), suggesting that their binding sites partially overlap in ubiquitin. Unlike the Tollip C2 and CUE domains, the N-terminal Tollip TBD region did not bind ubiquitin (data not shown). Interestingly, full-length Tollip bound ubiquitin with higher affinity than its individual domains, with a K_D of 423 nM (Table 1 and Fig. 3D), suggesting that cooperativity of the individual domains and oligomerization contribute to ubiquitin avidity. Taken together, we propose that two individual ubiquitin molecules can simultaneously bind Tollip through their C2 and CUE domains with both oligomerization and cooperativity of these domains playing a role in ubiquitin binding.

### Table 2

**Identification of critical residues for the Tollip C2 domain-ubiquitin interaction**

| Protein Construct | \(K_D\) (μM) | Fit | Fold |
|-------------------|-----------|-----|-----|
| Ubiquitin Wild-type | 1.08 × 10^{-5} | 0.83 | 1 |
| Ubiquitin L43A | 1.81 × 10^{-5} | 0.81 | 2 |
| Ubiquitin L44A | 1.25 × 10^{-5} | 1.61 | 1 |
| Ubiquitin E51A | 1.12 × 10^{-5} | 1.76 | 1 |
| Ubiquitin L71A | 2.83 × 10^{-5} | 2.30 | 3 |
| Ubiquitin K6A/L71A | 2.76 × 10^{-5} | 1.67 | 3 |
| Ubiquitin L71A/L73A | 2.76 × 10^{-5} | 2.54 | 3 |
| Tollip C2 | 3.54 × 10^{-6} | 2.07 | <1 |
| Tollip C2 R157A | 2.57 × 10^{-6} | 1.55 | <1 |
| Tollip C2 K162A | 1.83 × 10^{-5} | 0.82 | 2 |
| Tollip C2 L142A/K162A | 5.98 × 10^{-5} | 0.44 | 6 |

### Notes

- **Ubiquitin Inhibits the PtdIns(3)P Binding of Tollip**

- **Identification of critical residues for the Tollip C2 domain-ubiquitin interaction**

SPR-derived binding affinities of Tollip C2 domain for ubiquitin and their corresponding mutants designed based on HSQC titration analyses.
**Tollip CUE Domain Binds Ubiquitin Using Its Conserved MPF Motif**—To define the structural basis of the Tollip CUE domain-ubiquitin interaction and given the availability of the NMR resonance assignments of the Tollip CUE domain (21), we carried out HSQC measurements of the $^{15}$N-labeled Tollip CUE domain alone and with unlabeled ubiquitin (Fig. 4A). Several Tollip CUE domain resonances were perturbed by the presence of ubiquitin. Two ubiquitin-interacting regions are evident from the normalized chemical shift perturbation calculations (Fig. 4B). One region involves Ile-237, Asp-239, Met-240, Phe-241, and Asn-243 residues located between helices 1 and 2 in the Tollip CUE domain (Fig. 4B). Two of these residues, Met-240 and Phe-241, belong to the Met-Phe-Pro motif responsible for ubiquitin recognition in yeast CUE domains (20, 29). The second region includes C-terminal CUE domain residues, such as Met-270, Gly-271, and Glu-272. In contrast to the role of the Leu-Leu motif in ubiquitin interaction in yeast CUE domains (20, 29), the Tollip CUE domain Leu-Leu motif (amino acids 267–268) was not significantly perturbed by the presence of ubiquitin. To identify the binding surface, we generated a homology model of the Tollip CUE domain based on the structure of the Vps9p CUE domain, a protein that exhibits 23% identity with the Tollip CUE domain. The two ubiquitin-interacting regions are located at opposite ends of the CUE domain structure (Fig. 4C), inferring that it is possible that the Tollip CUE domain wraps around ubiquitin. We further quantitatively characterized the association of the Tollip CUE domain to ubiquitin using SPR analysis. Binding exhibited rapid association and dissociation rates and best fit the conformational change model with a resultant $K_D$ of $1.35 \mu M$ (Fig. 4D and Table 1). Thus, this result suggests that Tollip is able to simultaneously bind two ubiquitin molecules with affinities in the low micromolar range. Based on chemical shift perturbations induced by the Tollip CUE domain and using SPR analysis, we screened key ubiquitin interacting residues for Tollip CUE association. We found that ubiquitin Ile-44 mutation to alanine reduced Tollip CUE domain binding by about 43-fold, whereas mutation in Leu-69, close to Ile-44, reduced the affinity for the protein 3-fold (Table 3). Mutagenesis of neighbor residues in the tertiary structure of the protein (e.g. His-68) as well as in other NMR-perturbed residues (e.g. Leu-8) did not significantly alter Tollip CUE domain binding (Table 3). The structural integrity of ubiquitin upon mutation of Ile-44 was preserved based on a strong overlap on the CD traces (data not shown), indicating that mutation in ubiquitin affected Tollip CUE domain binding. Reciprocally, we introduced mutations in residues of the Tollip CUE domain that were perturbed by ubiquitin in HSQC titrations. Thus, replacement of Gly-271 with a valine residue decreased the affinity 53-fold and alanine mutations in residues Asp-239, Met-240, and Asn-243 reduced ubiquitin association 19-, 69-, and 9-fold, respectively, whereas mutation in Phe-241 did not affect ubiquitin binding (Table 3).
Accordingly, a triple mutant (D239A/M240A/N243A) of the Tollip CUE domain reduced its association to ubiquitin 78-fold (Table 3). Furthermore, mutations in ubiquitin-interacting residues did not alter the secondary structure of the Tollip CUE domain (data not shown). Consequently, the CUE domain conserved region that includes Met-240 and neighbor residues as well as the C-terminal region, including Gly-271, are compromised in ubiquitin ligation.

**DISCUSSION**

The results presented in this study demonstrate that phosphoinositide binding of Tollip is negatively modulated by ubiquitin and that this occurs by ubiquitin association to both Tollip C2 and CUE domains. This suggests the presence of a dual

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### TABLE 3

**Identification of critical residues for the Tollip CUE domain-ubiquitin interaction**

| Protein Construct | $K_D$ (nM) | Fit -Fold |
|-------------------|------------|-----------|
| Ubiquitin Wild-type | $1.35 \times 10^{-6}$ | 3.06 | 1 |
| Ubiquitin L8A | $2.00 \times 10^{-6}$ | 4.53 | 1 |
| Ubiquitin I44A | $5.80 \times 10^{-5}$ | 1.33 | 43 |
| Ubiquitin K63A | $3.10 \times 10^{-5}$ | 1.07 | 2 |
| Ubiquitin H68A | $2.00 \times 10^{-6}$ | 2.00 | 1 |
| Ubiquitin L69A | $3.80 \times 10^{-6}$ | 0.62 | 3 |
| Tollip CUE D239A | $2.52 \times 10^{-5}$ | 0.24 | 19 |
| Tollip CUE M240A | $9.33 \times 10^{-5}$ | 0.09 | 69 |
| Tollip CUE F241A | $1.05 \times 10^{-5}$ | 3.60 | <1 |
| Tollip CUE N243A | $1.16 \times 10^{-5}$ | 0.12 | 9 |
| Tollip CUE G271V | $7.11 \times 10^{-5}$ | 0.56 | 53 |
| Tollip CUE D239A/M240A/N243A | $1.05 \times 10^{-4}$ | 2.06 | 78 |

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mode of recognition for ubiquitin by Tollip. Whereas ubiquitin directly competes with PtdIns(3)P binding to the Tollip C2 domain, the action of ubiquitin on the CUE domain may reside in promoting dissociation of the Tollip dimer at its C terminus, which may reduce the affinity of Tollip for the phosphoinositide. On the basis of our findings, we propose that recruitment of Tollip to endosomal membranes, and formation of protein complexes with other adaptor proteins depend on its central C2 domain and the presence of PtdIns(3)P in endosomal membranes (Fig. 6). In the absence of polyubiquitinated cargo, Tollip could be partitioned in membrane-bound and membrane-free states, which depend on both the presence of PtdIns(3)P-rich domains and ubiquitin. The affinity of Tollip for PtdIns(3)P is 46 nM, which is 240-fold higher than its isolated C2 domain (18). This increment is not due to additional interactions of the protein via either TBD or CUE domains with PtdIns(3)P (data not shown); thus, oligomerization of Tollip provides high affinity and sensitivity to PtdIns(3)P levels at endosomal membranes. Because ubiquitin inhibits PtdIns(3)P binding, the Tollip-ubiquitin complex would remain membrane-free. Likewise, Tom1 is also an adaptor protein that associates to and acts downstream of Tollip and is also required to traffic ubiquitinated cargo, such as the IL-1R, to late endosomes (11). Tom1 contains two-ubiquitin binding domains, namely the VHS and GAT domains, and association of ubiquitin to the GAT domain prevents Tollip binding (10). Because Tom1 shares common ubiquitin binding properties with Tollip, we speculate that ubiquitin binding also prevents Tom1 recruitment to endosomal membranes via Tollip interactions and this may occur in the absence of polyubiquitinated cargo.

The question remains, what is the role of the Tollip-ubiquitin complex? Ubiquitin binds noncovalently to a variety of ubiquitin-binding domains, the majority of which associate to the hydrophobic patch around ubiquitin Ile-44 (1). Ubiquitin-binding domains cannot only interact with ubiquitin, but also associate with ubiquitinated proteins and be regulated by ubiquitination. Polyubiquitination of a protein can be a signal for its degradation by the proteasomal pathway; however, ubiquitin conjugation can also be a signal for changing protein localization or for regulation of a protein function (30). For example, the canonical activation of the transcription factor NF-κB through myeloid differentiation primary-response protein 88-dependent Toll-like receptor signaling requires activation of IRAK-4, which activates IRAK-1 by phosphorylation. Activated IRAK-1 associates with TRAF6, which in turn, activates its E3 ubiquitin ligase activity, that together with Ubc13 and Uev1A, catalyzes the Lys-63-linked polyubiquitination of IRAK1, NEMO, and TRAF6 itself (31). Thus, these chains act as a scaffold by helping the recruitment of ubiquitin-binding proteins including NEMO and TAB2/3. These associations facilitate the accumulation of NF-κB, required for the expression of pro-inflammatory proteins. We currently do not know whether the Tollip C2 and CUE domains bind polyubiquitin chains and, if so, whether they exhibit higher affinity than ubiquitin as demonstrated for the yeast CUE and UBA domains in vitro (19, 32). However, in this context, it is also possible that Tollip can be intracellularly partitioned and sequestered by interactions with

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**FIGURE 5.** Hydrodynamic properties of the Tollip CUE-ubiquitin complex. A, representative sedimentation velocity analysis of the Tollip CUE-ubiquitin complex. Sedimentation coefficient distribution of free Tollip CUE (s\textsubscript{top} = 1.64; green line), free ubiquitin (s\textsubscript{top} = 1.18; red line), and Tollip CUE-ubiquitin complex (s\textsubscript{top} = 1.73; black line). B, representative gel filtration analysis of ubiquitin (red), Tollip CUE domain (green), and ubiquitin:Tollip CUE domain (1:1 molar ratio; black) using a Superdex 75 column. Fractions of each of the peaks were analyzed using SDS-PAGE (top). C, summary of the results obtained from sedimentation velocity ultracentrifugation and analytical gel filtration analyses.

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**FIGURE 6.** A proposed model for the regulation of the endosomal membrane-associated Tollip. Tollip cycles between ubiquitin-free and -bound states in the absence of cargo proteins. Endosomal membrane binding of Tollip is mediated by the interaction of its C2 domain with PtdIns(3)P. The CUE domain mediates Tollip dimerization and this event and PtdIns(3)P binding is negatively regulated by ubiquitin. Of note, other ubiquitin-independent regions in Tollip (e.g. TBD) may also contribute to Tollip oligomerization.
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polyubiquitin chains of IRAK-1 via its C2 and CUE domains. This could be important as Tollip would be placed in proximity with IRAK-1 to modulate its activity in the absence of microbial products.

PtdIns(3)P is highly enriched in the cytosolic leaflet of the early endosomal membranes (33) and its function in these compartments is to recruit PtdIns(3)P-binding proteins. Formation of such protein-PtdIns(3)P complexes control membrane transport and dynamics such as intraluminal vesicle formation, receptor down-regulation, docking, fusion, and motility (34). Phosphoinositides are characterized for their rapid interconversion through the action of specific kinases and phosphatases. In mammals and yeast, PtdIns(3)P is produced from phosphatidylinositol by the class III PI 3-kinase. PtdIns(3)P synthesis is controlled by the small GTPase Rab5 because the class III PI 3-kinase acts as a Rab5 effector (34). In addition, PtdIns(3)P is dephosphorylated by lipid phosphatases (35). Thus, specific lipid kinases and phosphatases play a key role in the spatial and temporal regulation of PtdIns(3)P, which are crucial for tethering of adaptor protein complexes at endosomal membranes. From this study, we propose that ubiquitin binding to the Tollip C2 domain could represent a novel regulatory mechanism for engagement in both endosomal trafficking and other cytosolic commitments in the absence of cargo (Fig. 6).

The CUE domain is a helical module that belongs to the family of ubiquitin-binding domains (1). Whereas the Vps9p CUE domain binds ubiquitin in a dimeric conformation (20), the yeast Cue2 CUE domain predominantly interacts with ubiquitin as a monomer (29). Our oligomeric state analysis indicated that the dimeric Tollip CUE domain dissociated in the presence of ubiquitin suggesting that it could reduce its avidity for PtdIns(3)P leading to its release from endosomal membranes in the absence of cargo. Whereas the CUE domain mediates dimerization of Tollip (21) and GST-Tollip itself forms homodimers only (data not shown), we cannot exclude the possibility that additional regions in Tollip (i.e. TBD, but not the C2 domain) mediate oligomerization of the protein as reported from pulldown experiments (14). To better determine the oligomeric state of Tollip and because GST itself forms homodimers, we also tried to isolate a His-tagged version of Tollip, which resulted in degradation of the protein during the isolation process (data not shown). We found that the Tollip CUE domain exhibited ~10-fold higher affinity for ubiquitin than for that measured for the Tollip C2 domain (Table 3). This difference became evident from HSQC titrations of ubiquitin with the Tollip C2 and CUE domains. An 8-fold excess of C2 domain was required to visualize the interaction of ubiquitin with the Tollip C2 domain (Fig. 2D), 4-fold less than that used for monitoring the Tollip CUE domain interaction with ubiquitin (Fig. 3A). We established that Tollip binds to ubiquitin with a Kd of 423 nM; this is expected because, whereas ubiquitin-binding domains bind ubiquitin with low to moderate affinity as we see for both the individual C2 and CUE domains, the presence of multiple ubiquitin-binding domains cooperatively contribute to ubiquitin ligation (1). Cooperativity of neighbor ubiquitin-binding domains has recently been observed in the signal transducing adaptor molecule 2 (STAM2), one of the ESCRT-0 subunits that coordinate the proper function of the ESCRT protein complex responsible for committing ubiquitinated cargo to the multivesicular body pathway (40). STAM2 possesses two ubiquitin-binding domains, the VHS domain and the ubiquitin-interacting motif, which are separated from each other by a linker of 20 amino acids (41). Also, we observed that the affinity of Tollip for PtdIns(3)P is about 10-fold higher than ubiquitin. We reasoned that the relatively high intracellular concentration of free ubiquitin would com-
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pensate that difference and favor an equilibrium of free and membrane-bound Tollip states. In summary, our observations are in agreement with the general behavior of ubiquitin-binding proteins, which exhibit relatively moderate affinity for ubiquitin. However, this property is compensated by the presence of multiple ubiquitin-binding domains and, in some cases, by protein oligomerization (1). Altogether, our results place free ubiquitin as a novel modulator of the membrane targeting of Tollip, by triggering inhibition of PtdIns(3)P ligation and dissociation of the CUE domain dimers. Because ubiquitin-binding domains generally exhibit higher affinity for polyubiquitinated chains, the inhibitory function of ubiquitin should be balanced by an increment of polyubiquitinated cargo traffic, which in turn, will favor the commitment of Tollip, and other adaptor proteins, to sort cargo proteins through the endosomal pathway.

Acknowledgments—We thank Dr. Janet Webster for assistance during preparation of the manuscript. We also thank Dr. Shuyan Xiao and Kristen Fred for assistance with gel filtration.

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