Endophilin A1 is an SH3 domain-containing protein functioning in membrane trafficking on the endocytic pathway. We have identified the E3 ubiquitin ligase itch/AIP4 as an endophilin A1-binding partner. Itch belongs to the Nedd4/Rsp5p family of proteins and contains an N-terminal C2 domain, four WW domains and a catalytic HECT domain. Unlike other Nedd4/Rsp5p family members, itch possesses a short proline-rich domain that mediates its binding to the SH3 domain of endophilin A1. Itch ubiquitinates endophilin A1 and the SH3/proline-rich domain interaction facilitates this activity. Interestingly, itch co-localizes with markers of the endosomal system in a C2 domain-dependent manner and upon EGF stimulation, endophilin A1 translocates to an EGF-positive endosomal compartment where it co-localizes with itch. Moreover, EGF treatment of cells stimulates endophilin A1 ubiquitination. We have thus identified endophilin A1 as a substrate for the endosome-localized ubiquitin ligase itch. This interaction may be involved in ubiquitin-mediated sorting mechanisms operating at the level of endosomes.

Ubiquitination is a post-translational modification that involves the transfer of ubiquitin, an evolutionarily conserved 76 amino acid protein, to substrate proteins. The major specificity determinants of the ubiquitination machinery are the E3 ubiquitin ligases, which belong to two major classes, one that carries a RING (really interesting new gene) finger domain and another that carries a HECT (homologous to E6-AP C terminus) domain (1). RING finger E3s function as adaptors proteins, bringing the substrate to activated ubiquitin on E2-conjugating enzymes, whereas E3s of the HECT domain family bind to the activated ubiquitin and transfer it from the E2 to the substrate (2).

The classical view of ubiquitination is that it targets proteins for degradation by the 26 S proteasome (2). However, ubiquitin is now also recognized as a signal that directs plasma membrane proteins for endocytosis and regulates trafficking decisions that target endocytosed proteins for destruction in the lysosome (3–5). For example, the RING domain ligase cbl is recruited to the epidermal growth factor (EGF) receptor upon receptor activation by either EGF or transforming growth factor α (TGFα). Cbl mediates EGF receptor ubiquitination and endocytosis (6, 7). However, whereas EGF remains bound to the receptor in the more acidic environment of the endosome, TGFα rapidly dissociates upon internalization, the kinase activity of the receptor is attenuated and ubiquitination is lost (8, 9). The ligand-free receptor is targeted to the recycling pathway at the level of the endosome and is returned to the cell surface. In contrast, EGF remains bound to the receptor, which secures the kinase activity, and maintains receptor ubiquitination. The ubiquitinated EGF receptor is sorted to a trafficking pathway that leads to the lysosome where both ligand and receptor are degraded (9).

The mechanisms by which ubiquitination participates in endocytosis and trafficking decisions at the level of the endosome remain unclear. However, a growing body of evidence suggests that in addition to endocytic cargo, ubiquitination also regulates the protein machinery that controls cargo trafficking. For example, a functional ubiquitination machinery is required for down-regulation of the growth hormone receptor whereas ubiquitination of the receptor itself is dispensable (10). Moreover, in yeast, the α-factor receptor Ste-2, when expressed as an ubiquitin fusion protein, undergoes internalization without the need for additional ubiquitination (11). However, in the absence of the HECT domain ubiquitin ligase Rsp5p, internalization is impaired, suggesting that Rsp5p targets components of the trafficking machinery that regulate Ste-2 internalization (11). Ubiquitination thus has multiple roles in receptor down-regulation, acting both as a sorting signal on cargo and as a regulator of the trafficking machinery.

Recently, multiple classes of ubiquitin binding domains have been described (reviewed in Ref. 12). Interestingly, these domains are often found in components of the trafficking machinery in the endocytic pathway (13–16). These domains have been suggested to function as ubiquitin receptors, helping to sort ubiquitinated cargo to the endosomal pathway (17–20). Additionally, ubiquitin-binding domains may interact with ubiquitinated components of the trafficking machinery to allow for the formation of networks of protein interactions necessary for function of the machinery (12, 15).

Endophilin A1 is an SH3 domain-containing protein that functions in clathrin-mediated endocytosis (21–25). It was originally identified based on its interaction with proline-rich sequences of the endocytic proteins dynamin and synaptojanin (26–28). We previously screened a rat brain cDNA expression library with the SH3 domain of endophilin A1. In addition to known endophilin A1-binding partners, we identified the ger-
minal center kinase-like kinase, suggesting a role for endophilin A1 in the Jun kinase (JNK) signaling pathway (29). Another protein identified in this screen was the HECT domain E3 ubiquitin ligase itch/AIP4. Itch belongs to the Nedd4/Rsp5p family and was originally identified as a gene disrupted in the non-agouti-lethal 18H mice, which develop a spectrum of immunologic diseases including constant itching in the skin (30). Here, we have characterized the interaction of itch with endophilin A1. Our results suggest that itch may participate in protein trafficking at endosomes.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs and Mutagenesis**—The I.M.A.G.E. Consortium (LLNL) cDNA Clone 4838386 (31) encoding human itch was used as a template to generate various cDNA constructs by polymerase chain reaction (PCR) using Vent DNA polymerase (New England Biolabs) and the following primers: full-length itch, forward, 5′-GGAAGATTCAGATA- GTGCAACATACGAGCTCACAGT-3′, reverse, 5′-GGAAGTAC- CGGGCGCTGTATCTTCTGCACATCTTCTG-3′; itch ATC, amino acids 183–862, forward, 5′-GGAAGATCAATGCTGCAGCTAGATGTACGAGCTATGCTGTGTCCTCTG-3′, reverse, 5′-GGAAGGATTCGGCGCCGCTGTACGAGCTATGCTGTGTCCTCTG-3′; itch ATC2PRD, amino acids 230–862, forward, 5′-GGAAGATTCAGATAATGCTGCACTCATGGTTGCACATCTGCGT-3′, reverse, 5′-GGAAGGATTCGGCGCCGCTGTACGAGCTATGCTGTGTCCTCTG-3′; itch PRD, amino acids 183–235, forward, 5′-GGAAGTACGTATGCGGCTGATCAGGAGCTATGCTGTCCTCTG-3′, reverse, 5′-GGAAGGATTCCGGCGCTGTACGAGCTATGCTGTGTCCTCTG-3′. The resulting PCR products were subcloned into pFlag-CMV2 (Sigma-Aldrich), pGEX-4T-1 (Amersham Biosciences), or pEGFP-C2 (Clontech) to add FLAG, GST, and GFP tags, respectively. Ligase inactive itch (itch-CA) was produced by substituting Cys183 with Ala using the megaprimer procedure (32). Briefly, a forward primer encoding the mutation 5′-GAA-GTCTATCCGTTTACGGCATGCTG-3′ and a reverse primer 5′-GGAAGTACGTATGCGGCTGATCAGGAGCTATGCTGTCCTCTG-3′ were used in a first amplification reaction. The resulting PCR fragment was used as a megamer with the forward primer 5′-GGAAGTACGTATGCGGCTGATCAGGAGCTATGCTGTCCTCTG-3′ and a reverse primer 5′-GGAAGGATTCGGCGCCGCTGTACGAGCTATGCTGTGTCCTCTG-3′ to produce the full-length C830A mutant protein. GST-endophilin A1 constructs were described in a first amplification reaction. The resulting PCR fragment was used to produce the full-length itch protein identified in this screen was the HECT domain E3 ubiquitin ligase itch/AIP4. Our results suggest that itch may participate in protein trafficking at endosomes.

**In Vitro Ubiquitination Assays**—Recombinant ubiquitin was purchased from Sigma-Aldrich and ubiquitin activating enzyme (E1) and Ubch7 (E2) were purchased from Affinity Bioreagents. Endophilin A1, full-length itch, and itch CA proteins were produced as GST fusions and eluted from glutathione beads by thrombin cleavage. Ubiquitination reactions (25 μl) contained 10 μg of endophilin A1, ubiquitin (25 μM), ubiquitin-conjugating enzyme E1 (100 μM), ubiquitin-conjugating enzyme UbcH7 E2 (0.5 μM), 1.75 μg of itch or itch CA, and ATP (4 μM) in 50 mM Tris-Cl, pH 7.5, 2.5 mM MgCl2, and 1 mM dithiothreitol. The reactions were incubated for 90 min at 25 °C and then stopped by addition of 1/10 volume of SDS-PAGE loading buffer. The reaction mixtures were resolved by SDS-PAGE, transferred to nitrocellulose membranes and subjected to Western blot.

Endosome Purification—The preparation of early and late endosomal enriched fractions from cultured COS-7 cells was performed according to Gorvel et al. (35). Briefly, cells were washed three times with phosphate-buffered saline at 4 °C and then incubated on ice for 10 min in 1 ml of homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA). Cells were then homogenized in homogenization buffer containing protease inhibitors, and homogenized at 4 °C by five passages through a 27.5 Gauge needle. Homogenates were centrifuged for 15 min at 1000 × g at 4 °C, and the postnuclear supernatant (PNS) was collected. The PNS was brought to 40.6% sucrose by gradient centrifugation. The gradient was centrifuged for 40 min at 124,000 × g at 4 °C. Early and late endosomal fractions were collected at the 35%/30% sucrose interface and in the uppermost region of the 30% sucrose cushion, respectively.

**RESULTS**

**The Ubiquitin Ligase Itch Interacts with the SH3 Domain of Endophilin A1**—The screening of a rat brain expression library with a GST fusion protein coupled to the SH3 domain of endophilin A1 yielded 18 clones encoding potential endophilin A1 binding partners (29). Of these, two independent isolates encoded a rat homologue of the human E3 ubiquitin ligase itch/AIP4. To further validate the interaction, we co-transfected HEK-293 cells with a construct encoding full-length human itch with an N-terminal FLAG tag (FLAG-itch) and an untagged endophilin A1 construct. Immunoprecipitation of FLAG-itch with an anti-FLAG antibody led to co-immunoprecipitation of endophilin A1 (Fig. 1A). The FLAG antibody failed to immunoprecipitate endophilin A1 in protein extracts from cells transfected with endophilin A1 alone (Fig. 1A).

We next sought to determine the regions of endophilin A1 and itch involved in the interaction. Full-length FLAG-itch expressed in HEK-293 cells specifically bound to GST fusion proteins of full-length endophilin A1 and the isolated SH3 domain but failed to bind to endophilin A1 lacking the SH3 domain (data not shown). These experiments confirmed that the SH3 domain of endophilin A1 mediates the interaction with itch. To determine the SH3 binding domain of itch, GST-itch
fusion proteins were used in affinity selection assays from rat brain extracts. GST full-length itch bound to endophilin A1 with a near complete depletion of endophilin A1 from the extract (Fig. 1B, top panel). Deletion of the N-terminal C2 domain (itch ΔC2) did not affect the capacity of itch to bind endophilin A1, whereas further deletion of 44 amino acids containing the proline-rich domain (PRD) (itch ΔC2/ΔPRD) abolished the interaction (Fig. 1B, bottom panel). Identical results were obtained by co-immunoprecipitation analysis of FLAG-tagged itch deletions co-transfected with endophilin A1 (data not shown).

To determine if the itch/endophilin A1 interaction is direct, we performed an overlay assay. Extracts from HEK-293 cells expressing FLAG-Itch ΔC2, FLAG-Itch ΔC2/ΔPRD or mock-transfected cells were resolved by SDS-PAGE, transferred to nitrocellulose and incubated with GST-endophilin A1 or GST alone. The GST proteins were subsequently detected with an anti-GST antibody. GST-endophilin A1 bound to FLAG-Itch ΔC2 but not to FLAG-Itch ΔC2/ΔPRD (Fig. 1C). No binding was detected in the mock-transfected lane or with GST alone (Fig. 1C).

The 44 amino acids truncated in itch ΔC2/ΔPRD contain a short PRD, PSRPPRPSRP PPPPRP, which contains two sequences that resemble the consensus binding motif for the endophilin A1 SH3 domain (PXRPPPXP) (36). As detected by Coomassie Blue staining, endophilin A1 is the major protein affinity-selected from brain extracts by a GST-itch PRD fusion protein (Fig. 1D). Western blot analysis confirmed the identity of the isolated band as endophilin A1 (data not shown). These data suggest that the itch PRD interacts preferentially with endophilin A1 versus other SH3 domain bearing proteins in brain. However, amphiphysin and intersectin, which contain one and five SH3 domains, respectively, were both detectable by Western blot as itch PRD binding partners (data not shown).

**Itch Ubiquitinates Endophilin A1—HECT domain ubiquitin ligases of the Nedd4 family interact with their substrates through their WW domains, with recognition preferences for the motif PPX(Y/pY) (where pY = phosphorylated tyrosine) (37). Endophilin A1 does not contain such motifs but instead forms a stable complex with itch through its SH3 domain. Because of the unusual nature of this interaction for ubiquitin ligases, we sought to determine if endophilin A1 could serve as a substrate for the ubiquitin ligase activity of itch. We first utilized an *in vitro* ubiquitination assay with purified proteins. Recombinant endophilin A1 was incubated with purified mammalian ubiquitin-activating enzyme (E1), recombinant UbcH7 (E2), ubiquitin, and ATP. Wild-type (WT) full-length itch, an itch mutant, in which the active site cysteine in the HECT domain was mutated to an alanine (CA) were added to the reaction mixture. After incubation at room temperature, the reaction was resolved on SDS-PAGE and immunoblotted with antibody 1903 against endophilin A1. Ubiquitination of endophilin A1 in the presence of itch was detected by the presence of higher molecular weight bands, likely corresponding to the addition of one, two or three ubiquitin units (Fig. 2A). The ubiquitinated forms were detected at a level similar to the non-ubiquitinated protein suggesting a robust ubiquitination reaction (Fig. 2A). No ubiquitination of endophilin A1 was detected following incubation with itch CA (Fig. 2A).
Immunoprecipitated proteins were immunoblotted (IB) with Myc antibody and the same membrane was reprobed with GFP antibody. °a t2 5 WT CA or an inactive mutant (CA) (upper panel each). Cell lysates were processed for immunoprecipitation with anti-GFP antibody and immunoprecipitated proteins were immunoblotted (IB) with anti-Myc (right panel). The same membrane was reprobed with anti-GFP (left panel). C, HEK-293 cells were transfected with plasmids encoding Myc-tagged ubiquitin and GFP-endophilin A1, with (+) or without (−) plasmid encoding WT FLAG-itch (5 µg each). Cell lysates were processed for immunoprecipitation with anti-GFP antibody and immunoprecipitated proteins were immunoblotted (IB) with anti-Myc and the same membrane was reprobed with GFP antibody.

To further explore itch-induced endophilin A1 ubiquitination, we used a cell-based approach (38). HEK-293 cells were transfected with Myc-tagged ubiquitin and GFP-endophilin A1, with or without FLAG-itch. Cells were left untreated for 48 h and GFP-endophilin A1 was immunoprecipitated with an anti-GFP antibody. GFP Western blots revealed equal levels of GFP-endophilin A1 in all immunoprecipitates. The immunoprecipitated proteins were blotted with a monoclonal anti-Myc antibody to detect ubiquitination. Bands corresponding to mono- and poly-ubiquitinated GFP-endophilin A1 were detected only in cells ectopically expressing Flag-itch (Fig. 2B). Ubiquitinated forms of GFP-endophilin could also be detected in anti-GFP Western blots (Fig. 2B).

To determine if ubiquitination of endophilin A1 by itch depends on the SH3/PRD interaction, we examined for the ability of different FLAG-tagged itch constructs to induce endophilin A1 ubiquitination. Co-transfection of wild-type (WT) itch with GFP-endophilin A1 led to ubiquitination of GFP-endophilin A1 as detected by anti-Myc Western blot (Fig. 2C). Ubiquitinated bands were also detected with antibody 1903 against endophilin A1 (data not shown). No ubiquitination was seen following co-transfection of the itch CA mutant (Fig. 2C). Truncation of the N-terminal C2 domain, which does not alter the interaction between itch and endophilin A1 (Fig. 1B), did not affect endophilin A1 ubiquitination when compared with wild-type protein (Fig. 2C). However, further truncation of the PRD domain, leaving the WWs and the HECT domain intact, reduced the ability of itch to induce endophilin A1 ubiquitination (Fig. 2C). All of the FLAG-itch constructs were similarly expressed based on anti-FLAG Western blots and anti-GFP Western blots revealed similar levels of GFP-endophilin A1 in the immunoprecipitates (Fig. 2C). Thus, the SH3/PRD interaction appears to facilitate the ability of itch to ubiquitinate endophilin A1.

**Itch Localizes to an Endosomal Compartment**—The subcellular localization of itch has not been previously explored. We thus transfected COS-7 cells with GFP-tagged itch full-length constructs. At low and moderate levels of expression, GFP-itch accumulated around the nucleus, and exhibited a punctate distribution, which extended throughout the cell (Fig. 3 and 4, left panels). A similar distribution was observed using FLAG-itch (not shown). The perinuclear pool of itch overlapped substantially with markers of the trans-Golgi network (TGN) such as γ-adaptin (AP-1) and clathrin heavy chain (CHC) (arrows, Fig. 3, A and C). Although generally localizing to the same perinuclear region, GM130, a marker of the cis-Golgi, showed limited co-localization with GFP-itch (Fig. 3B, arrows). No significant overlap was observed with calnexin, a marker of the endoplasmic reticulum (Fig. 3D).

Additional itch staining was observed peripheral to the TGN in small, vesicular structures (see for example arrowheads, Fig. 3A). The vesicular structures were reminiscent of endosomes. The endosomal membrane system is organized in subcompartments characterized by the presence of specific proteins and phospholipids, and can be roughly divided into early, late and recycling endosomes (40). To determine more precisely the extent of the endosomal localization of itch, we compared its distribution to early endosomal antigen 1 (EEA1), a marker of early endosomes, and the cation-independent mannose-6-phosphate receptor (CI-MPR), which recycles between late endosomes and the TGN (41, 42). Extensive co-localization was observed between GFP-itch and CI-MPR, both in the perinuclear region (arrow) and in the vesicular structures (arrowheads) (Fig. 4A), suggesting that itch is present in the CI-MPR positive endosomal compartments as well as in the TGN. EEA1 also co-localized with itch (arrowheads, Fig. 4B), suggesting that itch is present in both the early and late compartments of the endosomal system. To label recycling endosomes, we performed uptake experiments with Cy3-transferrin (43). The labeled transferrin accumulated in tubule-like structures localized in the perinuclear region. Again, a partial overlap with
GFP-itch was observed (arrowhead, Fig. 4C). A similar approach was taken to label the lumen of the late endosome, using Texas Red-labeled EGF (TR-EGF), which is expected to accumulate in the late endosomal compartment (5). Following 20 min of uptake, TR-EGF accumulated in large puncta located inside the GFP-itch-containing tubules/vesicles (arrowhead, Fig. 4D). Taken together, these results suggest that itch is present in various endosomal subdomains.

Because the subcellular localization of itch was determined with exogenously expressed proteins, we sought to determine if endogenous itch also localized to endosomes. We obtained a monoclonal antibody against itch, which recognized both endogenous and overexpressed itch proteins in several cell-lines (data not shown). Unfortunately, the antibody did not work for immunofluorescence. We therefore performed an equilibrium centrifugation of COS-7 cell protein extracts on step gradient to enrich for endosomal compartments (35). Itch protein was detected predominantly in a CI-MPR positive late endosomal compartment although it was also enriched compared with crude cell lysates in an EEA1 positive early endosomal fraction (Fig. 5A). Consistently, fractionation of rat brain demonstrated strong staining for itch in an endosome enriched microsomal fraction (data not shown). Thus, endogenous itch appears associated with endosomal membranes.

The C2 domain of the ubiquitin ligases Nedd4 and Rsp5p mediate their interactions with membranes (44–47). In order to determine if the C2 domain contributes to itch endosomal localization, we transiently expressed a GFP fusion protein encoding an itch mutant lacking the N-terminal C2 domain (GFP-itch ΔC2). This construct demonstrated a cytosolic distribution (Fig. 5B) and similar results were obtained with FLAG-itch ΔC2 (data not shown). To determine if the C2 domain by
itself was sufficient to drive the subcellular localization of itch, we produced a vector encoding GFP-fused to the C2 domain alone (GFP-itch C2). This fusion protein was detected in the nucleus and the cytosol although there was a slight accumulation in a perinuclear pool (Fig. 5B). Only partial co-localization was observed between GFP-itch C2 and FLAG-itch (data not shown). These data thus suggest that the C2 domain of itch is necessary but not sufficient to drive its endosomal localization.

Endophilin A1 Translocates to the Endosome upon EGF Stimulation and Is Ubiquitinated—Whereas itch is localized predominantly to endosomes, endophilin A1 is diffusely distributed in the cytoplasm of transfected cells. However, upon EGF stimulation, endophilin A1 was found to co-localize with EGF receptors in endocytic vesicles (48). To further examine endophilin A1 trans-location upon EGF stimulation, we transfected COS-7 cells with GFP-endophilin A1 and followed the uptake of TR-EGF. After 1 h incubation with TR-EGF at 4 °C, EGF uptake was chased at 37 °C for 1 or 10 min. Although GFP-endophilin A1 showed only very limited co-localization with TR-EGF at 1 min (arrowheads, Fig. 6A), co-localization increased after TR-EGF uptake. By 10 min, concentration of GFP-endophilin A1 was visible in ring-like structures surrounding endocytosed EGF, demonstrating its localization to endosomes (arrowheads, Fig. 6B). To determine if itch and endophilin A1 co-localize at endosomes, we examined the distribution of GFP-endophilin A1 and Flag-itch in co-transfected cells following treatment with Alexa 647-EGF. After 10 min, endophilin A1 and itch were seen to co-localize in ring-like endosomal structures surrounding endocytosed Alexa 647-EGF (Fig. 6C). Thus, EGF treatment can stimulate the translocation of endophilin A1 to endosomal compartments containing itch. A perinuclear pool of GFP-endophilin A1 was sometimes ob-

![Figure 4. Localization of itch in endosomal compartments.](http://www.jbc.org/)

---

**Endophilin A1 Translocates to the Endosome upon EGF Stimulation and Is Ubiquitinated**—Whereas itch is localized predominantly to endosomes, endophilin A1 is diffusely distributed in the cytoplasm of transfected cells. However, upon EGF stimulation, endophilin A1 was found to co-localize with EGF receptors in endocytic vesicles (48). To further examine endophilin A1 trans-location upon EGF stimulation, we transfected COS-7 cells with GFP-endophilin A1 and followed the uptake of TR-EGF. After 1 h incubation with TR-EGF at 4 °C, EGF uptake was chased at 37 °C for 1 or 10 min. Although GFP-endophilin A1 showed only very limited co-localization with TR-EGF at 1 min (arrowheads, Fig. 6A), co-localization increased after TR-EGF uptake. By 10 min, concentration of GFP-endophilin A1 was visible in ring-like structures surrounding endocytosed EGF, demonstrating its localization to endosomes (arrowheads, Fig. 6B). To determine if itch and endophilin A1 co-localize at endosomes, we examined the distribution of GFP-endophilin A1 and Flag-itch in co-transfected cells following treatment with Alexa 647-EGF. After 10 min, endophilin A1 and itch were seen to co-localize in ring-like endosomal structures surrounding endocytosed Alexa 647-EGF (Fig. 6C). Thus, EGF treatment can stimulate the translocation of endophilin A1 to endosomal compartments containing itch. A perinuclear pool of GFP-endophilin A1 was sometimes ob-
Itch Ubiquitinates Endophilin

Fig. 5. Detection of itch in endosomal fractions and contribution of the C2 domain to localization. A, extracts from non-transfected COS-7 cells were processed by centrifugation on flotation sucrose step gradients in a procedure designed to maximize the separation of early and late endosomes. Equal protein aliquots of fractions corresponding to the crude lysate (sm), early (E) and late (L) endosomes, and soluble proteins (S) were processed for immunoblots with antibodies against the cation-independent mannose 6-phosphate receptor (CI-MPR), early endosomal antigen 1 (EEA1), or itch. B, COS-7 cells were transfected with plasmids encoding GFP-itch full-length, GFP-itch ΔC2, or GFP-itch C2, and fixed for imaging 15-h post-transfection. The scale bar represents 10 μm.

Itch binds to and ubiquitinates endophilin A1. We therefore examined if translocation of endophilin A1 to endosomes following EGF treatment was concomitant with its ubiquitination. HEP-293 cells were co-transfected with GFP-endo A1 and Myc-ubiquitin. After serum starvation, cells were treated with 100 ng/ml recombinant human EGF in serum-free media, followed by 10 min incubation at 37 °C. GFP-endo A1 was then immunoprecipitated with an anti-GFP antibody and blotted with an anti-Myc antibody to detect ubiquitination. A modest but reproducible increase in GFP-endo A1 ubiquitination was observed following a 10-min treatment with EGF. Thus, EGF can stimulate ubiquitination of endophilin A1, consistent with the observed translocation of endophilin A1 to endosomes following EGF treatment (Fig. 6D).

DISCUSSION

Endophilin A1 was first identified on the basis of the interaction of its SH3 domain with proline-rich motifs (26–28, 49). It was subsequently shown to function in endocytosis through its binding to synaptojanin and dynamin, two proteins of the clathrin-mediated endocytic machinery (21, 22, 26, 28). The SH3 domain of endophilin A1 binds preferentially to the motif PXRPPXPR, which is found in synaptojanin and dynamin (36). More recently, endophilin A1 was found to bind to similar motifs in two other proteins, germinal center kinase-like kinase (29) and apoptosis-linked gene 2-associated protein (50). Through these interactions, endophilin A1 is believed to function in JNK signaling and apoptosis. Here we have determined that the ubiquitin ligase itch uses a related motif to interact with endophilin A1 and that this interaction allows for endophilin A1 ubiquitination.

Several proteins have been identified as itch substrates including the membrane-associated proteins notch and occludin (38, 51); two nuclear proteins, the transcription factors NF-E2 and Jun b (52, 53); and two viral proteins, the Epstein-Barr virus latent membrane protein 2A and the adenovirus penton base protein (54, 55). However, little is known regarding the subcellular localization of itch relative to its substrates. The related ubiquitin ligase Nedd4 has been shown to associate with the apical plasma membrane of cells via calcium-dependent interaction of its C2 domain with the protein annexin XIIIb (45). This brings Nedd4 in proximity to its major target, ENaC (56). We report here that itch localizes through its C2 domain to the TGN and to endosomal membranes, where it co-localizes with endophilin A1 following EGF stimulation. The presence of C2 domains in HECT family ligases thus appears to play a general role in targeting these proteins to substrates at specific membrane subcompartments in cells. Moreover, our results on the C2 domain-dependent localization of itch to endosomes are relevant to the recently discovered role of itch in the regulation of EGF receptor trafficking. Itch has been reported to bind through its WW domains to the RING domain E3 ligase cbl (39, 57) and overexpression of itch can induce a down-regulation of EGF receptor signaling that is additive to cbl-induced down-regulation (39). Interestingly, overexpression of an itch ΔC2 construct does not cause down-regulation of EGF receptor signaling (39). Thus, the C2 domain-dependent localization of itch to endosomes appears critical to its ability to regulate EGF receptor trafficking.

In addition to interacting with itch, cbl has also been recently described to bind through its C terminus to the SH3 domain of CIN85 (cbl-interacting protein of 85 kDa) (48, 58). Interestingly, CIN85 binds through a PRD to endophilin and formation of the cbl/CIN85/endophilin complex is required for endocytosis of both EGF and c-Met receptors (48, 58). The coupling of this complex to the EGF receptor through cbl likely provides the mechanism for the EGF-dependent targeting of endophilin A1 to endosomes observed in our study. Once this complex reaches the endosome, itch could bind through its WW domain to cbl (39, 57) and through its PRD, it could displace CIN85 in binding to the SH3 domain of endophilin A1. Through ubiquitination of either or both proteins, itch could contribute to the down-regulation of EGF receptor by regulating its trafficking to the lysosome (59). Our observation that endophilin is ubiquitinated following treatment with EGF is consistent with this model. Moreover, EGF fails to ubiquitinate endophilin A1 in cells overexpressing the itch CA ligase inactive mutant (data not shown), further suggesting that itch is the ligase that mediates the EGF effect. However, a general lowering in ubiquitinated proteins was observed in these cells, precluding a definitive demonstration of the involvement of endogenous itch in EGF-induced endophilin A1 ubiquitination. Nevertheless, endophilin A1 and itch both appear to have key roles in EGF receptor trafficking. Interestingly, a very recent study in yeast has demonstrated that Rsp5p, a yeast homologue of itch, binds to Slal and Rvs167, described as yeast homologues of CIN85 and endophilin (60). Moreover, Rsp5p monoubiquitinites Rvs167 (60). Thus, the substrate recognition and ubiquitination of endophilin by itch appears to be conserved throughout eukaryotic evolution.

Recent studies indicate that activation of the EGF receptor increases the amount of ubiquitinated proteins that are associated with endosomes (14). This increase cannot be fully accounted for by the presence of ubiquitinated receptors themselves, implying that EGF receptor activity may lead to ubiquitination of proteins that are involved in endosome function (5, 14). Our data suggest that one possible mechanism for this accumulation is provided by the translocation of endocytic proteins to endosomes following EGF stimulation, bringing potential ubiquitination substrates such as endophilin in close proximity to endosome-resident ligases such as itch. The po-
**Fig. 6.** Endophilin A1 translocates to endosomes following EGF treatment and is ubiquitinated. Texas-Red-EGF (TR-EGF) was endocytosed into COS-7 cells transfected with GFP-tagged endophilin A1 (Endo A1) for: A, 1 min and B, 10 min. The colocalization of endophilin A1 (green) with TR-EGF (red) is revealed in the blended images (blend) and with higher magnification (blend (3×)). Arrowheads point to examples of co-localization. C, Alexa 647-EGF was endocytosed for 10 min in COS-7 cells transfected with GFP-tagged endophilin A1 and FLAG-tagged itch. The colocalization of endophilin A1 (green) with Alexa 647-EGF (blue) and itch (red) is revealed in the blended image (blend) and with higher magnification (blend (3×)) as white color. Arrowheads point to examples of co-localization. Scale bars represent 10 and 3.3 μm for the magnified images. D, HEK-293 cells, transfected with plasmids encoding Myc-tagged ubiquitin and GFP-endophilin A1 were serum-starved and then treated with 100 ng/ml EGF in serum-free media for 0 or 10 min. Soluble cell lysates were processed for immunoprecipitation with GFP antibody and the immunoprecipitated proteins were immunoblotted (IB) with a Myc antibody (right panel). The same membrane was reprobed with a GFP antibody (left panel).
tential role and functional significance of such an ubiquitina-
tion cascade is emerging with the discovery of ubiquitin-bind-
ning domains in endocytic proteins such as epsins, eps15 and Hrs (15). Interestingly, Hrs accumulates on endosomes at mi-
crodromes that are coated with a flat clathrin lattice (17, 61, 62). Through binding to ubiquitin, Hrs recruits ubiquitinated, lysosomal-targeted receptors, including EGF receptor, to these
microdomains (17, 61, 62). Retention of the ubiquitinated cargo
precedes their incorporation to internal endosomal vesicles,
which are generated from inward invagination of the endoso-
mal membrane and are a step on the pathway to the lysosome
(61). Ubiquitination of endophilin A1 could position the protein
with Hrs in these endosomal microdomains. Interestingly,
through lipid interactions, endophilin can contribute to the
formation of membrane curvature (63, 64). Further studies are
needed to fully appreciate the role of ubiquitinated endophilin
A1 at endosomes.

Acknowledgments—We thank Dr. Satoshi Waguri and Dr. John
Bergeron for antibodies. We also thank Drs. Fiona Bedford, Lara Fallon,
and Ted Fon for useful discussion and advice, and Elaine De Heuvel
and Jacynte Philie for excellent technical assistance. We are also
grateful to the individual members of the McPherson laboratory.

REFERENCES


14. Itch Ubiquitinates Endophilin

15. Acknowledgments

16. REFERENCES

17. Acknowledgments

18. REFERENCES

19. Acknowledgments

20. REFERENCES

21. Acknowledgments

22. REFERENCES

23. Acknowledgments

24. REFERENCES

25. Acknowledgments

26. REFERENCES

27. Acknowledgments

28. REFERENCES

29. Acknowledgments

30. REFERENCES

31. Acknowledgments

32. REFERENCES

33. Acknowledgments
The HECT Domain Ligase Itch Ubiquitinates Endophilin and Localizes to the trans -Golgi Network and Endosomal System
Annie Angers, Antoine R. Ramjaun and Peter S. McPherson

doi: 10.1074/jbc.M309934200 originally published online December 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309934200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 28 of which can be accessed free at http://www.jbc.org/content/279/12/11471.full.html#ref-list-1