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Ubiquitin-Interacting Motifs of Epsin Are Involved in the Regulation of Insulin-Dependent Endocytosis

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Epsin is a key molecule in receptor-mediated endocytosis. Epsin is phosphorylated and ubiquitinated, and these post-translational modifications are necessary for the regulation of endocytosis. Since human Epsin (hEpsin) has two ubiquitin-interacting motifs (UIMs), we investigated the roles of these UIMs in endocytosis. hEpsin formed a complex with ubiquitinated proteins but did not bind to monoubiquitin. Neither of the two UIMs of hEpsin alone was sufficient to form a complex with ubiquitinated proteins: both UIMs were necessary. Mutations of Asp209 and Asp210 to Ala in UIM (hEpsinDA) abolished the binding activity of hEpsin to ubiquitinated proteins. However, hEpsinDA interacted with Eps15, POB1, and AP-2, which are involved in receptor-mediated endocytosis, as efficiently as wild-type hEpsin. Expression of hEpsinDA in CHO-IR cells affected neither the binding of insulin to nor insulin-dependent autophosphorylation of its receptor. Expression of wild-type hEpsin inhibited the internalization of insulin, whereas that of hEpsinDA did not. These results suggest that the UIM motifs of hEpsin interact with proteins modified with ubiquitin, and that the complex formation is involved in insulin-dependent receptor endocytosis.

Key words: endocytosis, Epsin, ubiquitin, UIM.

Abbreviations: AP, adaptor protein; EGF, epidermal cell–derived growth factor; EGFR, EGF-receptor; EH, Eps15 homology; ENTH, Epsin N-terminal homology; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; HA, hemagglutinin-1; hEpsin, human Epsin; Hrs, hepatocyte growth factor–regulated tyrosine kinase substrate; IP, immunoprecipitation; IRS, insulin receptor substrate; MBP, maltose-binding protein; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; POB1, Partner of RalBP1; RT-PCR, reverse-transcriptase-PCR; UIM, ubiquitin-interacting motif.

Epsin has been identified as a binding protein of the EH domains of Eps15, POB1, and intersectin (*1–4*). The N-terminal region of Epsin has an evolutionarily conserved domain called ENTH that binds to phosphatidylinositol-4,5-bisphosphate (*5–7*). The central region of Epsin is characterized by the presence of eight repeats of the Asp-Pro-Trp (DPW) motif, which binds to AP-2 (*1*). The C-terminal region of Epsin contains a Leu-Val-Asp-Leu-Asp (LVDLD) sequence, which binds directly to clathrin (*8*), and three repeats of the Asn-Pro-Phe (NPF) motif, which is known to constitute the binding sequence of the EH domains of Eps15 and POB1 (*1–3*, *9*). Overexpression of these Epsin domains or motifs inhibits the clathrin-dependent internalization of the receptors for EGF, insulin, and transferrin (*1*, *3*), implying that Epsin regulates endocytosis by interacting with lipids, AP-2, clathrin, and EH domain-containing proteins.

In addition to these characteristic regions, Epsin contains ubiquitin-interacting motifs (UIMs). The UIM was initially identified in the ubiquitin-binding region of the PRN10 subunit of the 26S proteasome (*10*) and this motif was first described as a peptide sequence consisting of a highly conserved A-x-x-Ala-x-x-x-Ser-x-x-Ax core, where A represents a hydrophobic residue, Ax is an acidic residue, and X is any amino acid (*11*). UIM has been found in not only Epsin but also in other endocytotic proteins, including Eps15, Eps15R, Hrs, Vps27, and Hse1/STAM (*11*). Deletions of UIMs and point mutations of conserved UIM residues in Hrs and Vps27 compromise the transport of ubiquitinated cargo *in vivo* (*12*, *13*). However, how the UIMs are involved in the regulation of receptor-mediated endocytosis is largely unknown.

Ubiquitination is a post-translational modification resulting in the covalent attachment of ubiquitin through its C-terminal Gly to a Lys residue in a target protein (*14*). Polyubiquitination, the attachment of multimeric chains of ubiquitin, leads to the proteolytic destruction of proteins in proteasomes when Lys48 of ubiquitin is the site of chain formation (*14*). However, ubiquitin chains formed through Lys63 are not involved in protein degradation, but rather in a variety of other processes, including DNA repair, transcription, translation, protein-trafficking, and virus-binding (*15*). Monoubiquitination of the cytoplasmic tail of several proteins has been shown to be important for the internalization of these proteins (*16*). Furthermore, it has been reported that EGFR is monoubiquitinated at multiple sites and that a single ubiquitination is sufficient for receptor internalization (*17*, *18*). Thus, understanding how cells recognize and sort ubiquitinated proteins is of general importance.

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Ent1p, which is a counterpart of Epsin in *Saccharomyces cerevisiae*, also has two UIMs (*19*). Monoubiquitination in α-factor receptor is sufficient for its internalization (*20*) and functions as a cargo-sorting signal in the multivesicular body pathway (*21*, *22*). Wild-type *ent1* is able to rescue a defect of α-factor internalization in a mutant in which the Ent proteins are nonfunctional, whereas the $ent^{uim\Delta}$ mutant, in which the UIMs are lacking, is not (*19*). Ent1p binds to monoubiquitin and its UIMs are both sufficient and necessary for interaction with ubiquitin (*19*). However, although the UIMs from Epsin, Eps15, and Hrs in mammalian cells have also been shown to bind directly to monoubiquitin *in vitro* (*23*), the dissociation constants are relatively high (in the range of 0.15–1 mM) (*24*). Furthermore, the roles of UIMs of Epsin in endocytosis in mammalian cells have not yet been examined experimentally.

We have demonstrated that the small GTP-binding protein Ral and its downstream molecules, RalBP1 and POB1, are involved in receptor-mediated endocytosis of EGF and insulin (*3*, *25*). Furthermore, we have found that Eps15 and Epsin bind directly to the EH domain of POB1 (*2*, *3*), and that the phosphorylation of Epsin by cdc2 kinase during mitosis inhibits endocytosis (*26*). Therefore, the signaling from Ral to Eps15 and Epsin through RalBP1 and POB1 regulates receptor-mediated endocytosis. In this study, we show the involvement of UIMs of Epsin in insulin-dependent receptor endocytosis through binding to ubiquitinated proteins.

MATERIALS AND METHODS

Materials and Chemicals—CHO-IR (insulin receptor–overexpressing Chinese hamster ovary cells), HepG2 (human hepatocarcinoma cells), and SHSY5Y (human neuroblastoma cells) cells were kindly provided by Drs. Y. Ebina (Tokushima University, Tokushima), Y. Matsuura (Osaka University, Suita), and M. Matsumoto (Hiroshima University, Hiroshima), respectively. Hygromycin-resistant CHO-IR cells that stably express human Epsin (hEpsin) or its mutants were propagated as described (*3*). GST- and MBP-fusion proteins were purified from *Escherichia coli*. The anti-Myc antibody was generated by 9E10 cells. [^{125}I]insulin was purchased from Amersham Pharmacia Biotech, Inc. (Buckinghamshire, UK). The anti-ubiquitin, anti-HA, anti-FLAG, FK1 (anti–polyubiquitinated protein monoclonal antibody), PY20 (anti-phosphotyrosine monoclonal antibody), and anti-GFP antibodies were from MBL, Inc. (Nagoya), Roche Diagnostics GmbH (Mannheim, Germany), Sigma Corp. (St. Louis, MO, USA), BIOMOL® International L.P. (Plymouth Meeting, PA, USA), MP Biomedicals Corp. (Irvine, CA, USA), and Invitrogen Corp. (Carlsbad, CA, USA), respectively. Other materials and chemicals were from commercial sources.

Plasmid Construction—pEF-BOS-Myc/hEpsin, pEF-BOS-Myc/hEpsin-(1-205), pEF-BOS-Myc/hEpsin-(204-458), pEF-BOS-Myc/hEpsin-(433-551), pEGFP/hEpsin, pGEX-2T/POB1-(126-227)(POB1-EH), pGEX-2T/Eps15-(1-330)(Eps15-EH), and pGEX-2T/app were constructed as described (*2*, *3*, *26*). Standard DNA-recombination techniques were used to construct the following plasmids: pMAL-c2/hEpsin-(1-289), pGEX-KG/hEpsin-(184-198), pGEX-KG/hEpsin-(209-223), pGEX-KG/hEpsin-(207-227), pGEX-KG/hEpsin-(184-223) [UIM(Epsin)], pGEX-KG/hEpsin-(184-223)DA [UIM (EpsinDA)], pGEX-KG/Eps15-(852-866), pGEX-KG/Eps15-(878-892), pGEX-KG/Eps15-(852-896), pGEX-KG/Hrs-(259-273), pGEX-KG/Hrs-(257-277), pEGFP-hEpsinDA, pEF-BOS-Myc/hEpsinDA, and p3XFLAG-CMV®-10/hEpsin. Some of the constructs in these plasmids were made by digesting the original plasmids with restriction enzymes and inserting the fragments of interest into the vectors. Other constructs were made by inserting fragments generated by PCR into the vectors. The entire sequence of each PCR product was determined and the structures of all plasmids were confirmed by restriction analyses.

Complex Formation of Epsin with Ubiquitinated Proteins in Intact Cells—COS cells (100-mm-diameter dishes) expressing FLAG-hEpsin or Myc-hEpsin together with HA-ubiquitin were lysed in 300 μl of NP-40 buffer [20 mM Tris/HCl (pH 7.5), 135 mM NaCl, and 1% Nonidet P-40®] containing inhibitor cocktail (20 μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 5 mM sodium orthovanadate, 50 mM β-glycerophosphate, and 10 mM *N*-ethylmaleimide). The lysates (400 μg of protein) were immunoprecipitated with the anti-FLAG or anti-Myc antibody and the immunoprecipitates were probed with the anti-FLAG, anti-Myc, or anti-HA antibody. In double IP analyses, COS cells (100-mm-diameter dishes) expressing GFP-hEpsin and HA-ubiquitin were lysed in 300 μl of NP-40 buffer containing inhibitor cocktail. After the lysates (400 μg of protein) were immunoprecipitated with the anti-GFP antibody, the immunoprecipitates were resuspended in 30 μl of Laemmli's sample buffer and boiled at 100°C for 8 min. Three hundred microliters of RIPA buffer [10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS] containing inhibitor cocktail was added to the sample, and the sample was centrifuged at 2,300 × *g* for 2 min. Then the supernatant was immunoprecipitated with the anti-GFP antibody again, and the immunoprecipitates were probed with the anti-HA and anti-GFP antibodies.

Complex Formation of Epsin with Ubiquitinated Proteins In Vitro—The lysates (200 μg of protein) of COS cells (100-mm-diameter dishes) expressing HA-ubiquitin were incubated with 1 μM MBP-hEpsin-(1-289) or various deletion mutants of GST-hEpsin and precipitated with amylose resin and glutathione-Sepharose 4B, respectively, by centrifugation. The precipitates were probed with the anti-HA, anti-MBP, or anti-GST antibodies.

Binding of Epsin to POB1, Eps15, and α-Adaptin—To assess the binding of Epsin to POB1 and Eps15, overlay assays were performed as described (*2*, *3*, *27*). The lysates (20 μg of protein) of COS cells expressing Myc-hEpsin or Myc-hEpsinDA were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with 1 ml of overlay buffer [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 30 mg/ml BSA, 1 mM DTT, and 0.1% Tween-20] containing 1–2 nM GST-POB1-(126-227), GST-Eps15-(1-330), or GST for 12 h at 4°C. The membranes were then washed and probed with the anti-GST antibody.

To examine the binding of Epsin to α-adaptin, the lysates (300 μg of protein) of COS cells expressing Myc-hEpsin or Myc-hEpsinDA were incubated with 1 μM

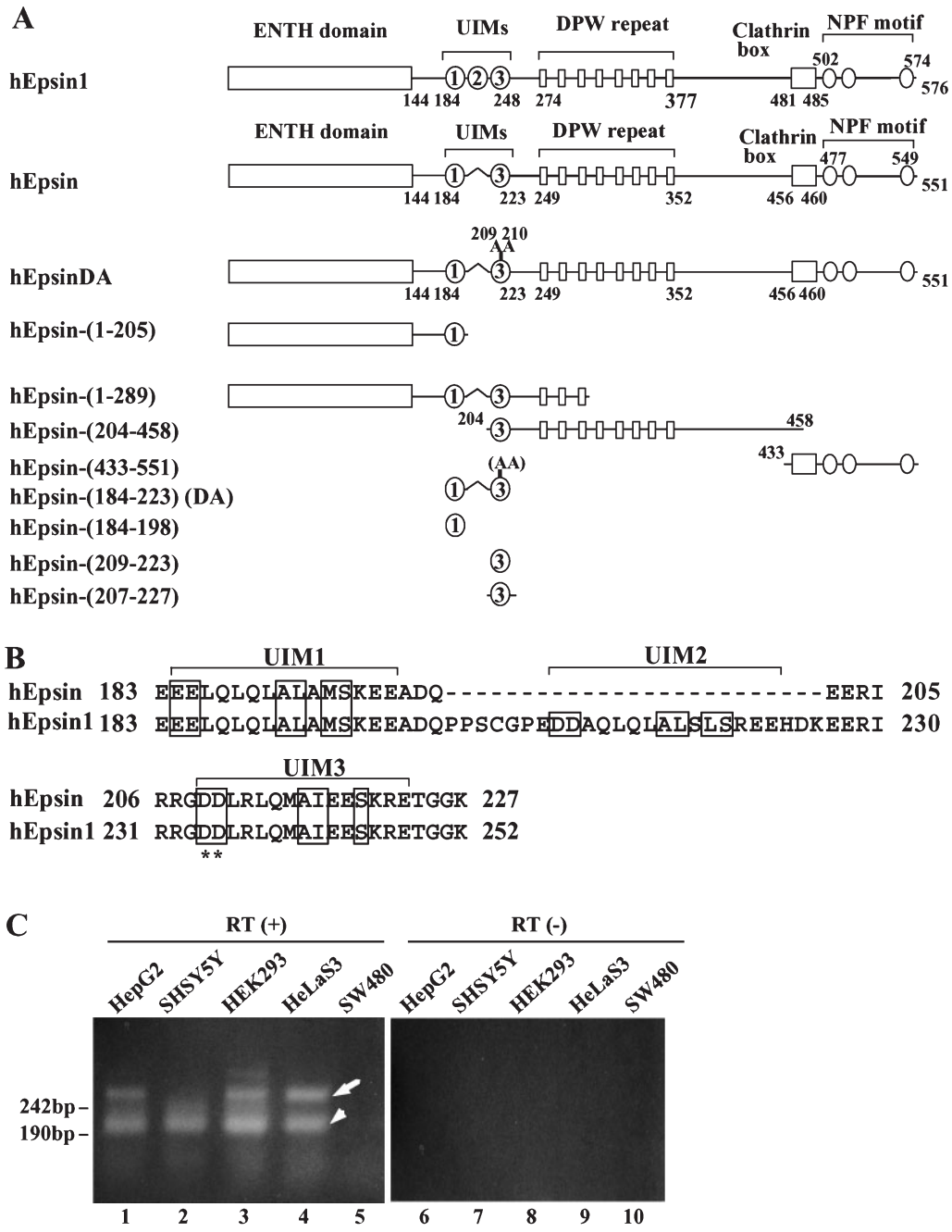
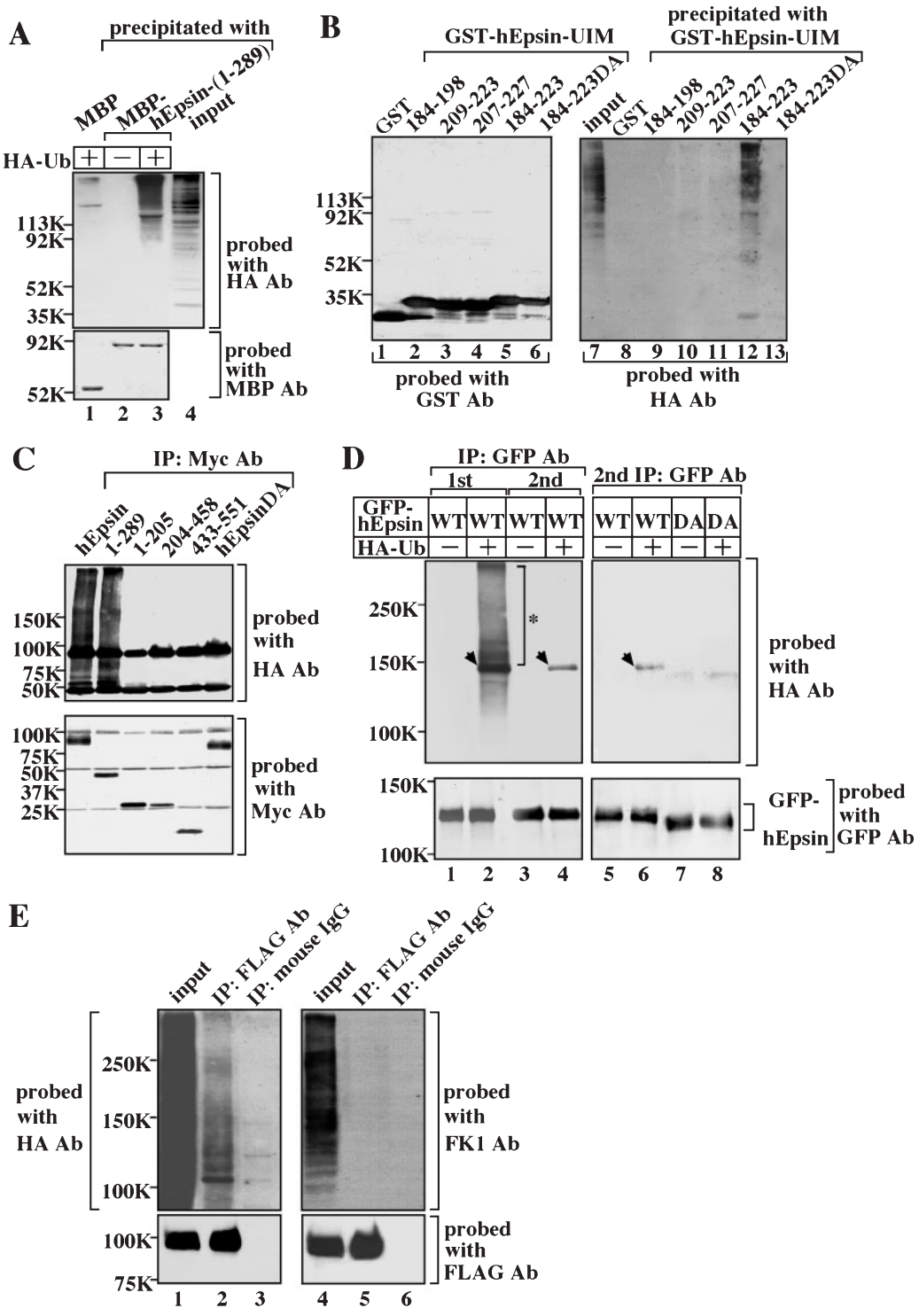


Fig. 1. **Structure of hEpsin.** (A) Schematic representation of hEpsin and its deletion mutant constructs. (B) Alignment of the amino acid sequences of the UIM motifs of hEpsin and hEpsin1. Boxed amino acids are highly conserved among various UIMs. Asp209 and Asp210, which were altered to Ala in hEpsinDA and hEpsin-(184-223)DA, are marked by asterisks (*). (C) Expression of hEpsin and hEpsin1 in various cell lines. RT-PCR analyses were performed with specific primers for hEpsin and hEpsin1 using total RNA from HepG2 (lanes 1 and 6), SHSY5Y (lanes 2 and 7), HEK293 (lanes 3 and 8), HeLaS3 (lanes 4 and 9), and SW480 (lanes 5 and 10) cells. Control reactions were performed without reverse-transcriptase (lanes 6–10) [RT (–)]. The arrow and the arrowhead indicate the positions of the PCR products derived from hEpsin1 and hEpsin, respectively.

GST-fused appendage domain of α-adaptin (GST-app) or GST immobilized on GSH-Sepharose 4B for 1 h at 4°C. After centrifugation, the precipitates were probed with the anti-GST and anti-Myc antibodies.

Insulin Binding and Internalization Assay—The binding and the internalization activities of insulin in CHO-IR cells were determined as described previously (*2*, *3*). Confluent wild-type CHO-IR cells and CHO-IR cells stably expressing Myc-hEpsin or its mutants (35-mm-diameter dishes) were incubated with 100 pM [^{125}I]insulin (1–2 × 10^3 cpm/pmol) for 5 h at 4°C. Internalization was initiated by adding warm binding medium [Ham's F12 medium containing 50 mM Hepes/NaOH (pH 7.4) and 1 mg/ml BSA] at 37°C after the cells had been washed with ice-cold PBS three times. At various times, the medium was removed and the cells were washed with acidic wash-



ing buffer (0.2 M acetic acid and 0.5 M NaCl). The acid-stripped cells were lysed with PBS containing 1% Triton X-100 and 0.1% SDS. Insulin binding was expressed as the sum of surface-bound and internalized [^{125}I]insulin and the rate of the internalization of insulin was expressed as the percentage of internalized [^{125}I]insulin relative to the sum of surface-bound and internalized [^{125}I]insulin.

Transferrin Internalization Assay—The transferrin internalization assay was performed as described previously (*3*, *28*). Control CHO-IR cells and CHO-IR cells stably expressing Myc-hEpsin grown in 24-well plates were washed with and incubated in assay medium [McCoy's 5A medium salts containing 20 mM Hepes/NaOH (pH 7.4), 26 mM $NaHCO_3$, and 1 mg/ml BSA] for 1 h at 37°C. The cells were washed with assay medium and incubated in assay medium supplemented with 25 nM [^{125}I]transferrin for the indicated periods. After incubation, the plates were placed on ice and washed with PBS containing Ca^{2+} and Mg^{2+} (PBS+) and then with the acidic wash-

ing buffer for 5 min at 4°C. The cells were then washed three times with cold PBS+ and solubilized in 1 N NaOH, and the radioactivities of the lysates were counted in a gamma counter. Cell-associated radioactivity after the acid wash represents the internalized transferrin during the incubation at 37°C. The amount of the surface transferrin receptor was determined by incubating cells with 25 nM [^{125}I]transferrin on ice for 2 h followed by seven washes with cold PBS+, and the radioactivities of the lysates solubilized in 1 N NaOH were counted in a gamma counter. Internalization of transferrin was determined as the ratio of internalized transferrin/surface transferrin binding. The nonspecific binding was less than 5% of the total binding in the presence of a large excess of nonradioactive diferric transferrin. The surface transferrin binding was constant over the course of the experiment. The concentration of transferrin used was sufficient to saturate the surface transferrin receptors rapidly.

RT-PCR—Total RNA from cultured cells was prepared using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biosciences, Buckinghamshire, UK). Total RNA (2 μg) was used for the random-primed-reverse-transcription reaction. The cDNA fragment including the last UIM motif in hEpsin1 and hEpsin was amplified by 50 cycles of PCR. The primers used were 5′-GCACG-CGCTCAAGACCA-3′ and 5′-AGCCGCAGGTCATCCC-3′.

RESULTS

UIMs in Human Epsin—In a previous study, we isolated a protein that binds to the EH domain of POB1 and designated it human Epsin (hEpsin) (*2*, *3*). At present, five human Epsin family members are known: hEpsin, hEpsin1, hEpsin2a, hEpsin2b, and hEpsin3. Database analyses indicate that hEpsin and hEpsin1 are splicing valiants. The product lacking a 25-amino-acid segment including the middle UIM is hEpsin, and that containing it is hEpsin1 (Fig. 1, A and B). Therefore, hEpsin and hEpsin1 contain two and three UIMs, respectively. RT-PCR analyses showed that hEpsin is expressed in human cells lines, including HepG2 cells (hepatocellular carcinoma cells), SHSY5Y cells (neuroblastoma cells), HEK293 cells (embryonic kidney cells), and HeLaS3 cells (uterus carcinoma cells), whereas hEpsin1 is expressed in HepG2, HEK293 cells, and HeLaS3 cells, but not in SHSY5Y cells (Fig. 1C). Neither hEpsin nor hEpsin1 was expressed in SW480 cells (colorectal cancer cells).

Interaction of hEpsin with Ubiquitinated Proteins—Various UIMs have been shown to bind to polyubiquitin or monoubiquitin (*24*), but the possibility that Epsin interacts with ubiquitinated proteins through UIM was not previously tested experimentally. When HA-ubiquitin was expressed in COS cells, numerous ubiquitinated proteins were detected using the anti-HA antibody (Fig. 2A, lane 4). MBP-hEpsin-(1-289), which includes two UIMs, but not MBP precipitated ubiquitinated proteins (Fig. 2A, lanes 1 and 3). To determine the minimum region of hEpsin that binds to ubiquitinated proteins, various deletion mutants, including mutants lacking one or both UIMs, were purified as GST-fusion proteins. GST-hEpsin-(184-223) formed a complex with ubiquitinated proteins, but GST-hEpsin-(184-198), GST-hEpsin-(209-223), and GST-hEpsin-(207-227) did not (Fig. 2B). Asp209 and Asp210 are conserved amino acids in various UIMs (Fig. 1B) (*23*). Mutations of both Asps to Ala in GST-hEpsin-(184-223) impaired the ability of hEpsin to bind to ubiquitinated proteins (Fig. 2B, lane 13). These results clearly indicate that neither of the UIMs alone is sufficient for the binding of hEpsin to ubiquitinated proteins: both of the UIMs are required. We did not observe detectable binding of the UIMs of Epsin to monoubiquitin itself (data not shown).

To confirm these results in intact cells, various deletion mutants of Myc-hEpsin were expressed together with HA-ubiquitin in COS cells. Myc-hEpsin-(1-205) and Myc-hEpsin-(204-458) contain the first and second UIM, respectively. Numerous proteins were detected using the anti-HA antibody in the Myc-hEpsin immune complexes. Under the same conditions, Myc-hEpsin-(1-289) formed a complex with ubiquitinated proteins, but Myc-hEpsin-(1-205), Myc-hEpsin-(204-458), Myc-hEpsin-(433-551), and Myc-hEpsinDA did not (Fig. 2C). Therefore, UIMs are required for the complex formation between hEpsin and ubiquitinated proteins in intact cells. To confirm that the high-molecular-mass proteins were indeed ubiquitinated proteins bound to Epsin, the immunoprecipitates were treated with 3.3% SDS and boiled to disrupt noncovalent

Fig. 2. **Interaction of Epsin with ubiquitinated proteins.** (A) Complex formation of hEpsin and ubiquitinated proteins *in vitro*. The lysates of COS cells expressing HA-ubiquitin (lanes 1 and 3) or empty vector (lane 2) were precipitated with MBP (lane 1) or MBP-Epsin-(1-289) (lanes 2 and 3) immobilized on amylose resin. The precipitates were probed with the anti-HA (upper panel) and anti-MBP (lower panel) antibodies. One percent of input was used as authentic ubiquitinated proteins (lane 4). Ub, ubiquitin; Ab, antibody. (B) Minimal region of hEpsin that binds to ubiquitinated proteins. The lysates of COS cells expressing HA-ubiquitin were precipitated with 1 μM GST (lanes 1 and 8), GST-hEpsin-(184-198) (lanes 2 and 9), GST-hEpsin-(209-223) (lanes 3 and 10), GST-hEpsin-(207-227) (lanes 4 and 11), GST-hEpsin-(184-223) (lanes 5 and 12), or GST-hEpsin-(184-223)DA (lanes 6 and 13). The precipitates were probed with the anti-GST (left panel) and anti-HA (right panel) antibodies. One percent of input was used as authentic ubiquitinated proteins (lane 7). (C) Complex formation of Epsin and ubiquitinated proteins in intact cells. The lysates of COS cells expressing Myc-hEpsin or its mutants with HA-ubiquitin were immunoprecipitated with the anti-Myc antibody and probed with anti-HA (upper panel) or anti-Myc (lower panel) antibody. (D) Double IP assay. After the lysates of COS cells expressing GFP-hEpsin (lanes 1–6) or GFP-hEpsinDA (lanes 7 and 8) with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) HA-ubiquitin were immunoprecipitated with the anti-GFP antibody (1st IP), the immunoprecipitates were resuspended in Laemmli's sample buffer, heated at 100°C and diluted with RIPA buffer. Then the samples were immunoprecipitated again with the anti-GFP antibody (2nd IP), and the immunoprecipitates were probed with the anti-HA or anti-GFP antibody. The asterisk indicates ubiquitinated proteins. Arrows indicate ubiquitinated hEpsin. (E) Interaction of hEpsin with polyubiquitinated proteins. The lysates of COS cells expressing FLAG-hEpsin and HA-ubiquitin were immunoprecipitated with the anti-FLAG antibody (lanes 2 and 5) or with nonimmune mouse Ig (lanes 3 and 6), and probed with the anti-HA antibody (left upper panel, lanes 1–3), FK1 (right upper panel, lanes 4–6), or anti-FLAG antibody (lower panel, lanes 1–6). Ten percent of input was used as authentic ubiquitinated proteins (lanes 1 and 4).

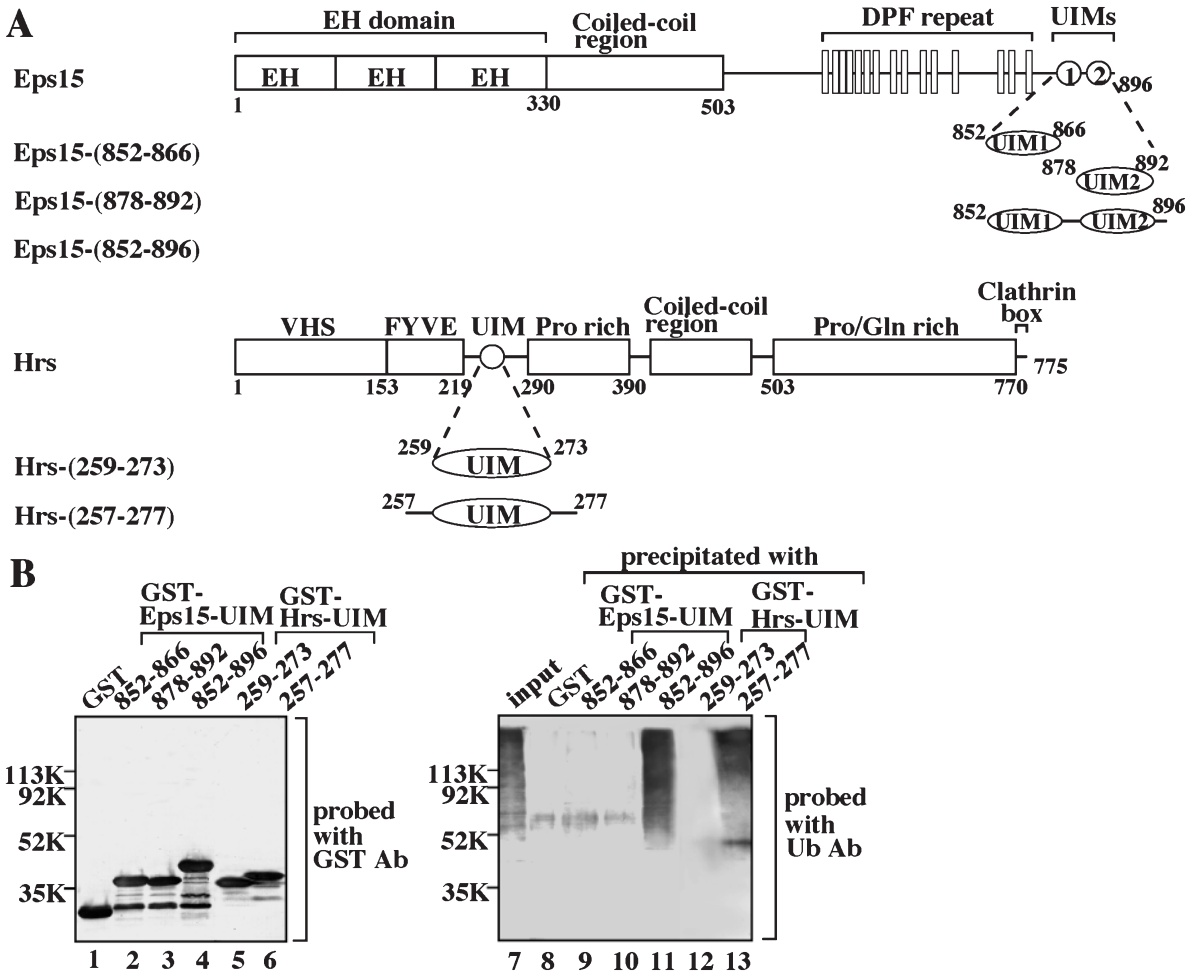


Fig. 3. **UIMs of Eps15 and Hrs.** (A) Schematic representation of Eps15 and Hrs. (B) Binding of UIMs of Eps15 and Hrs to ubiquitinated proteins. The lysates of COS cells expressing HA-ubiquitin were precipitated with 1 μM GST (lanes 1 and 8), GST-Eps15-(852-866) (lanes 2 and 9), GST-Eps15-(878-892) (lanes 3 and 10), GST-Eps15-(852-896) (lanes 4 and 11), GST-Hrs-(259-273) (lanes 5 and 12), or GST-Hrs-(257-277) (lanes 6 and 13). The precipitates were probed with the anti-GST (left panel) or anti-ubiquitin (right panel) antibodies. One percent of input was used as authentic ubiquitinated proteins (lane 7).

protein-protein interactions. This treatment indeed abolished most of anti-HA immunoreactive bands, and only the band of about 140 kDa was observed (Fig. 2D). Therefore, the slowly migrating band of GFP-hEpsin at around 140 kDa reflects covalent modification with ubiquitin, which is consistent with the previous observation that Epsin1 is ubiquitinated (*23*). Under the same conditions, covalent modification of GFP-hEpsinDA with ubiquitin was greatly reduced (Fig. 2D). These results are also consistent with the reports that UIM-containing proteins are ubiquitinated in a UIM-dependent manner (*29*).

To examine whether hEpsin binds to polyubiquitinated proteins, we used an anti-ubiquitin antibody, FK1, that is known to recognize polyubiquitin (Fig. 2E) (*30*, *31*). FK1 did not recognize the anti-HA immunoreactive bands that were found in the hEpsin immune complexes (Fig. 2E). Therefore, hEpsin does not bind to polyubiquitinated proteins.

Interaction of UIMs of Eps15 and Hrs with Ubiquitinated Proteins—We next examined the binding of UIMs of other endocytotic proteins to ubiquitinated proteins. Eps15 has two UIMs and Hrs has one UIM (Fig. 3A). GST-Eps15-(852-896), but not GST-Eps15-(852-866) and GST-Eps15-(878-892), precipitated ubiquitinated proteins (Fig. 3B). These results suggest that two UIMs are necessary for the complex formation of Eps15 with ubiquitinated proteins. However, this was not the case for Hrs. GST-Hrs-(257-277) could bind to ubiquitinated proteins although GST-Hrs-(259-273) did not (Fig. 3B). These results indicate that the addition of a few amino acids to the N- and C-termini of UIM in Hrs results in retention of the binding activity. Therefore, the binding activity of the UIM-containing proteins to ubiquitinated proteins is not always dependent on the number of UIMs.

Interaction of hEpsinDA with POB1, Eps15, and AP-2—The EH domains of POB1 and Eps15 bind to the Asn-Pro-Phe (NPF) sequence of Epsin and AP-2 binds to Epsin through the Asp-Pro-Trp (DPW) sequence (*1*, *3*). To examine whether mutations of UIMs in hEpsin affect the binding of hEpsin to these proteins, Myc-hEpsin and Myc-hEpsinDA were expressed in COS cells. Overlay analyses were performed using GST-POB1-EH and GST-Eps15-EH as probes. GST-POB1-EH and Eps15-EH bound to both Myc-hEpsin and Myc-hEpsinDA (Fig. 4A,

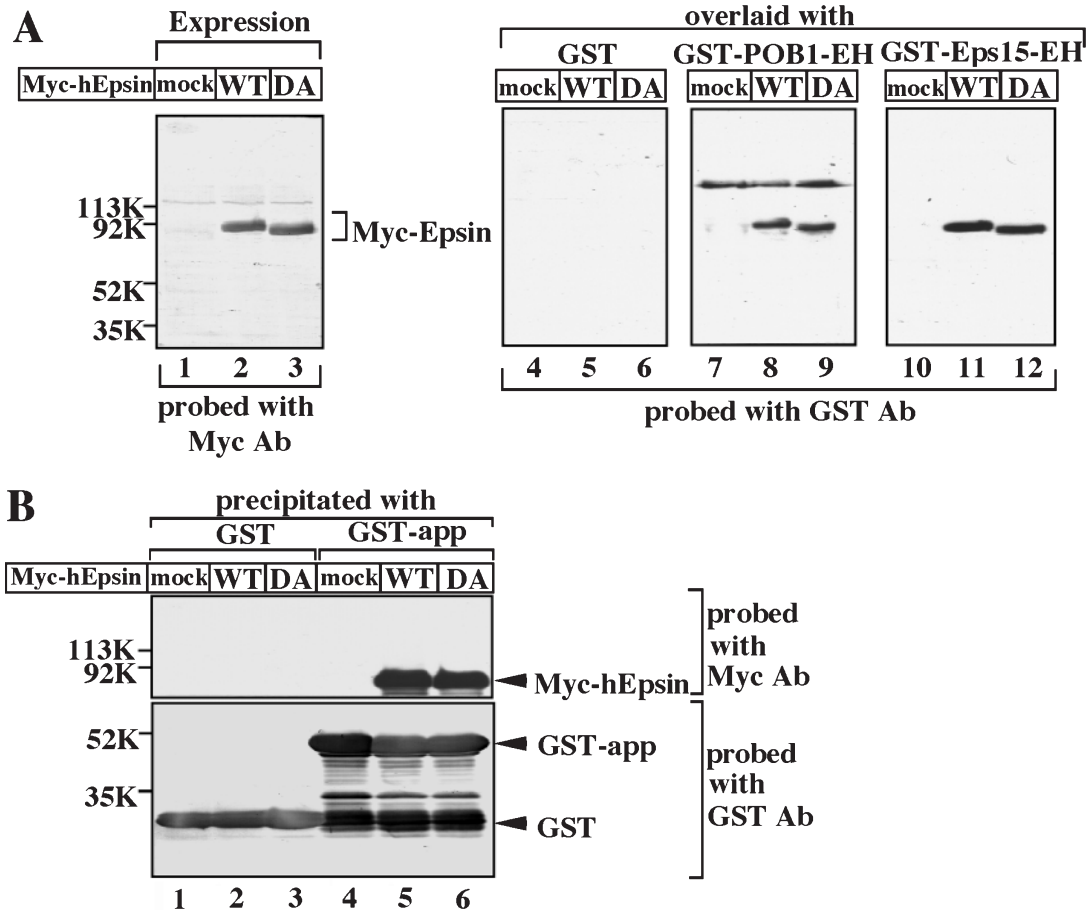


Fig. 4. **Binding of hEpsinDA to POB1, Eps15, and AP-2.** (A) Interaction of hEpsinDA with the EH domains. The lysates of COS cells expressing empty vector (lanes 1, 4, 7, and 10), Myc-hEpsin (lanes 2, 5, 8, and 11), and Myc-hEpsinDA (lanes 3, 6, 9, and 12) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, and then overlaid with 2 nM GST (lanes 4–6), 1 nM GST-POB1-EH (lanes 7–9), or 2 nM GST-Eps15-EH (lanes 10–12). Expression of Myc-hEpsin or Myc-hEpsinDA was confirmed using the anti-Myc antibody (lanes 1–3). WT, wild-type hEpsin; DA, hEpsinDA. (B) Interaction of hEpsinDA with AP-2. The lysates of COS cells expressing empty vector (lanes 1 and 4), Myc-hEpsin (lanes 2 and 5), or Myc-hEpsinDA (lanes 3 and 6) were precipitated with 1 μM GST (lanes 1–3) or GST-app (lanes 4–6) immobilized on GSH-Sepharose 4B. The precipitates were probed with the anti-Myc (upper panel) or anti-GST (lower panel) antibodies.

lanes 7–12). GST alone did not bind to Myc-hEpsin (Fig. 4A, lanes 4–6). The appendage domain of α-adaptin in AP-2 was purified as a GST-fusion protein (GST-app). GST-app, but not GST, precipitated both Myc-hEpsin and Myc-hEpsinDA (Fig. 4B). These results indicate that the mutations of UIM in hEpsin do not impair the binding of Epsin to POB1, Eps15, or AP-2.

Effects of UIM of hEpsin on Endocytosis—We previously showed that hEpsin is involved in receptor-mediated endocytosis of insulin in studies using CHO-IR cells that overexpress the insulin receptor (*2*, *3*). To examine the roles of UIM of hEpsin in insulin-dependent endocytosis, we established CHO-IR cells stably expressing Myc-hEpsin or Myc-hEpsinDA (Fig. 5A). Insulin bound to wild-type CHO-IR cells at 4°C, and expression of Myc-hEpsin or Myc-hEpsinDA did not affect the insulin-binding activity (Fig. 5B). Insulin stimulation of these stable transformants resulted in tyrosine autophosphorylation of the insulin receptor β-subunit similar to that seen in control CHO-IR cells (Fig. 5A). These results indicate that the UIMs of hEpsin are not involved in the binding of insulin to its receptor. To examine the roles of the UIMs of Epsin in insulin receptor internalization, CHO-IR cells expressing Myc-hEpsin or Myc-hEpsinDA were incubated with [^{125}I]insulin for 5 h at 4°C and further incubated without insulin at 37°C (Fig. 5C). As reported previously (*2*), about 35% of prebound insulin underwent internalization by 7.5 min in the control CHO-IR cells. Expression of Myc-hEpsin resulted in about a 50% reduction of the internalization, while that of hEpsinDA did not affect insulin internalization (Fig. 5C). These results imply that the expression of hEpsin blocks endocytosis perhaps through sequestration of ubiquitinated proteins into non-functioning complexes during endocytosis, whereas hEpsinDA does not affect internalization because it does not bind to ubiquitinated proteins. Therefore, the binding of hEpsin to ubiquitinated proteins is important for insulin-dependent receptor endocytosis.

Using the same CHO-IR cells stably expressing Myc-hEpsin, we examined the effects of hEpsin on endocytosis of transferrin and its receptor. The transferrin receptor is a constitutively recycled protein and internalized in a ligand-independent manner (*32*, *33*). Control CHO-IR cells and CHO-IR cells stably expressing Myc-hEpsin exhib-

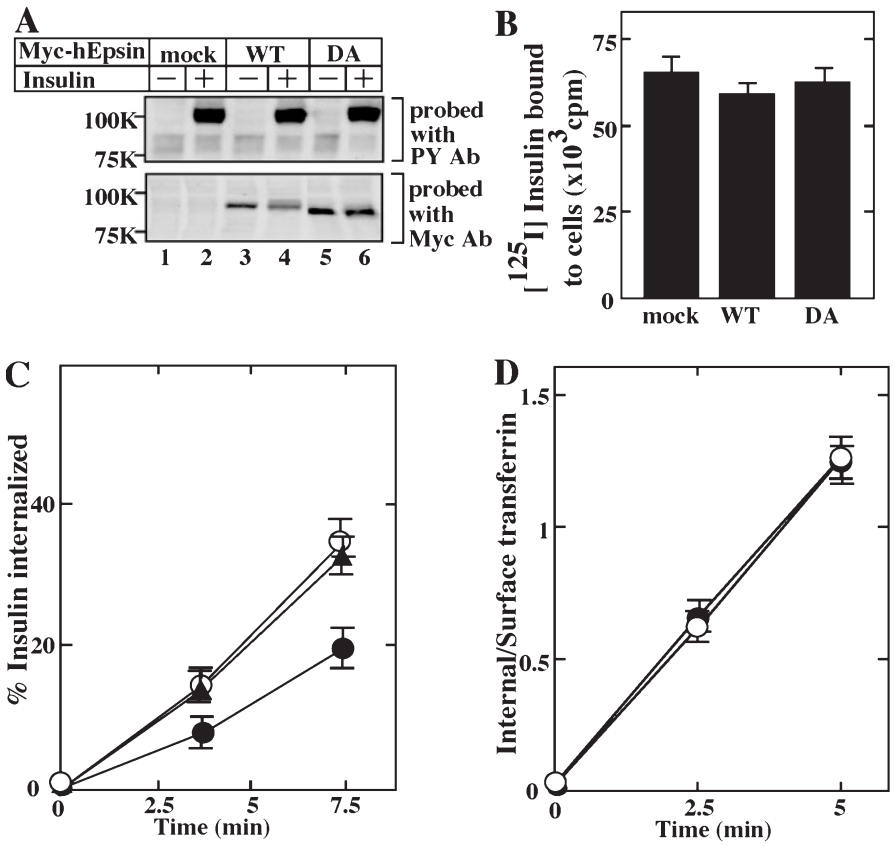


Fig. 5. **Involvement of UIM of hEpsin in the internalization of insulin.** (A) Autophosphorylation activity of the insulin receptor. Control CHO-IR cells (lanes 1 and 2), CHO-IR cells stably expressing Myc-hEpsin (lanes 3 and 4), or CHO-IR cells stably expressing Myc-hEpsinDA (lanes 5 and 6) were incubated with (+) or without (–) 100 nM insulin for 10 min at 37°C. Aliquots of the lysates of these cells were probed with the anti-phosphotyrosine or anti-Myc antibody. PY, phosphotyrosine. (B) Insulin-binding activity. Control CHO-IR cells, CHO-IR cells stably expressing Myc-hEpsin, or CHO-IR cells stably expressing Myc-hEpsinDA were incubated with 100 pM [^{125}I]insulin for 5 h at 4°C, and then washed and lysed. The radioactivity bound to the cells was measured with an autogamma counter. When a large excess of non-labeled insulin was added, the radioactivity bound to the cells was reduced to less than 10% of that in the absence of non-labeled insulin. The results shown are means ± SE of five independent experiments. (C) Internalization of insulin. Control CHO-IR cells (open circles), CHO-IR cells expressing Myc-hEpsin (closed circles), or CHO-IR cells expressing Myc-hEpsinDA (closed triangles) were incubated with 100 pM [^{125}I]insulin for 5 h at 4°C, and further incubated without insulin at 37°C. The rate of internalization of insulin was expressed as the percentage of internalized [^{125}I]insulin relative to total cell associated-[^{125}I]insulin after binding for 5 h. The results shown are means ± SE of five independent experiments. (D) Internalization of transferrin. Control CHO-IR cells (open circles) or CHO-IR cells expressing Myc-hEpsin (closed circles) were incubated with 25 nM [^{125}I]transferrin and the transferrin internalization activities of the cells were measured. The results shown are means ± SE of five independent experiments.

ited the same internalization rate constant for transferrin, which indicates the fraction of surface transferrin receptors internalized per minute (Fig. 5D). Therefore, involvement of hEpsin in receptor-mediated endocytosis may be depend on the receptor types.

DISCUSSION

Our results in this study show that the two UIMs of hEpsin bind to ubiquitinated proteins non-covalently and that this binding is necessary for insulin-dependent endocytosis. These conclusions are supported by the following findings. First, neither of two UIMs in hEpsin was by itself sufficient for the binding of hEpsin to ubiquitinated proteins. Moreover, hEpsinDA, in which two conserved Asp located in the second UIM were substituted by Ala, lost the ability to bind to ubiquitinated proteins. Second, hEpsinDA did not lose the ability to bind to Eps15, POB1, or the α-adaptin of AP-2, which are important proteins in receptor-mediated endocytosis. Thirdly, the expression of hEpsinDA did not affect the internalization of insulin under conditions in which the expression of wild-type hEpsin inhibited it.

It is thought that the overexpression of hEpsin interferes with the functions of proteins involved in endocytosis through the recruitment of binding partners. Several possible explanations for why hEpsinDA does not affect the internalization of insulin are conceivable. The binding of ubiquitinated proteins to hEpsin may regulate the binding of hEpsin to Eps15, POB1, or AP-2 in intact cells, although hEpsinDA is able to bind to these binding partners *in vitro* as efficiently as wild-type hEpsin. Alternatively, hEpsinDA may fail to interact with other Epsin-binding proteins that we did not examine, such as intersectin or clathrin. Further, the complex formation between hEpsin and ubiquitinated proteins may be essential for insulin-dependent receptor internalization in our assay system.

The affinity of the single UIM of Hrs and monoubiquitin is in the range of 150–300 μM, whereas that of the two UIMs of Eps15 and monoubiquitin is lower (*24*). We could not detect binding of hEpsin to monoubiquitin (data not shown), which is not consistent with previous observations (*23*). Therefore, there is little correlation between the number of UIMs in proteins and the binding activity of proteins to monoubiquitin. We showed that hEpsin, a splicing variant without the second UIM of hEpsin1, binds to ubiquitinated proteins and that additional amino acids adjacent to the UIM are required for the interaction with ubiquitinated proteins in the case of Hrs. These results suggest that the three-dimensional structures of UIM, including the surrounding amino acids, might be important for the selectivity for binding to monoubiquitin, ubiquitin chains, or ubiquitinated proteins.

The molecular mechanism for the endocytosis of EGFR is the most extensively studied example of endocytosis

(*34*). Upon stimulation with EGF, EGFR undergoes ligand-induced dimerization and activation of its cytoplasmic kinase domain, resulting in tyrosine auto-phosphorylation and ubiquitination by c-Cbl, an E3 ubiquitin ligase (*34*). The auto-phosphorylation of Tyr1045 accelerates the binding of c-Cbl to EGFR, followed by the phosphorylation of c-Cbl by EGFR and ubiquitination of EGFR at the plasma membrane (*35*). Although it is not clear whether c-Cbl simply mediates monoubiquitination or multiple monoubiquitination of EGFR, Eps15 and/or Epsin might bind to monoubiquitin on internalizing receptors and localize to the vicinity of the Nedd4 ubiquitin ligase, which in turn is activated by EGFR signals and induces monoubiquitination of Eps15. Upon the monoubiquitination of Eps15 itself, intramolecular interactions between the UIMs of Eps15 and its own monoubiquitination site would lead to its dissociation from EGFR. The sorting of ubiquitinated receptors into the inner vesicles of late endosomes or multivesicular bodies depends on the recognition of these receptors by UIMs of another set of UIM-containing proteins, including Hrs and TSG101.

Like EGF, insulin activates the tyrosine kinase activity of its receptor (*36*). IRS is a key molecule in the insulin-signaling cascade. Upon stimulation with insulin, IRS binds to the insulin receptor and is tyrosine-phosphorylated, and consequently forms a signaling complex with several SH2 domain–containing proteins, including PI3K, Grb2, and Fyn. Although it is unclear whether the insulin receptor undergoes ligand-dependent ubiquitination, it has been shown that an adapter protein with Pleckstrin homology and Src homology 2 domains (APS) recruits c-Cbl to the insulin receptor and causes it to dissociate from the c-Cbl associating protein (CAP) in an insulin-dependent manner (*37*). Therefore, c-Cbl may function as an E3 ubiquitin ligase for the insulin receptor. Alternatively, IRS may be monoubiquitinated or multiply monoubiquitinated instead of the insulin receptor, because the protein levels of IRS are regulated by a ubiquitin and proteasome degradation pathway (*38*, *39*).

Several membrane receptors are reported to be ubiquitinated in a ligand-dependent manner (*40–43*). Since the transferrin receptor is a constitutively recycled protein and internalized in a ligand-independent manner (*32*, *33*), the transferrin receptor may not be ubiquitinated. This could be the reason why the expression of hEpsin affects the internalization of insulin but not transferrin. However, overexpression of Epsin2 has been shown to inhibit the internalization of transferrin (*44*). In these experiments hEpsin was highly expressed transiently and the endocytosis of the transferrin receptor was observed immunohistochemically, while we expressed hEpsin moderately and measured the internalization of transferrin quantitatively using radioisotopes. The discrepancy between those results and ours might be due to the differences in the experimental conditions. Furthermore, it has been shown that the transferrin receptor is polyubiquitinated (*45*). Whether the transferrin receptor is monoubiquitinated and whether the UIM of hEpsin is involved in the regulation of endocytosis of the transferrin receptor remain to be clarified.

Epsin itself is ubiquitinated (*23*) and UIM promotes its ubiquitination (*29*). However, all of the studies in which these findings were made utilized UIM mutants that would simultaneously block ubiquitin-binding as well as ubiquitination, making it impossible to determine whether the observed effects were due to one or both of these activities. Therefore, identification of the site of ubiquitination in hEpsin would make it possible to clarify which of the abilities, ubiquitin-binding or ubiquitination, indeed alters the functions. Although we show that the UIMs of hEpsin are necessary for insulin internalization, it remains to be clarified whether they are required for further sorting of the insulin receptor to lysosomes for degradation or to the Golgi apparatus for recycling. Further studies will be necessary to elucidate the roles of UIM and the ubiquitination of hEpsin itself in the insulin-dependent receptor internalization.

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