Clathrin-independent endocytosis of ubiquitinated cargos

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Plasma membrane receptors can be endocytosed through clathrin-dependent and clathrin-independent pathways. Here, we show that the epidermal growth factor (EGF) receptor (EGFR), when stimulated with low doses of EGF, is internalized almost exclusively through the clathrin pathway, and it is not ubiquitinated. At higher concentrations of ligand, however, a substantial fraction of the receptor is endocytosed through a clathrin-independent, lipid raft-dependent route, as the receptor becomes ubiquitinated. An ubiquitination-impaired EGFR mutant was internalized through the clathrin pathway, whereas an EGFR/ubiquitin chimera, that can signal solely through its ubiquitin (Ub) moiety, was internalized exclusively by the non-clathrin pathway. Non-clathrin internalization of ubiquitinated EGFR depends on its interaction with proteins harboring the Ub-interacting motif, as shown through the ablation of three Ub-interacting motif-containing proteins, eps15, eps15R, and epsin. Thus, eps15s and epsin perform an important role in the internalization step of endocytosis.

Materials and Methods

Transfection and Biochemical Studies. Transfections were performed by using Lipofectamine or Oligofectamine (Invitrogen). For biochemical experiments, cells were serum-starved and then stimulated with EGF (100 ng/ml, unless otherwise indicated) at 37°C. Lysis, immunoprecipitation, and immunoblotting were performed as described in ref. 7. Plasmids, pharmacological agents, and antibodies are described in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Gene Silencing. Gene silencing of eps15, eps15R, or epsin was obtained by pSUPER vectors encoding specific targeting sequences. Two distinct small interfering RNAs/targets were tested, with comparable results. Stable clones were obtained by cotransfection with a plasmid carrying puromycin resistance. Three independent clones were analyzed with similar results. Silencing of clathrin heavy chain was by transient transfection of two different short interfering RNA oligos (which yielded comparable results) directed against the two previously described sequences (8, 9). Further details are in Supporting Materials and Methods.

Immunofluorescence and Internalization Studies. Immunofluorescence, internalization of rhodamine-labeled ligands, 125I-EGF or 13A9 antibody were performed as described in ref. 4. At the single cell level, endocytosis was monitored both by rhodamine-EGF and by anti-EGFR 13A9 antibody (which does not interfere with EGFR internalization) with comparable results (only one of the two procedures is shown in the pictures). Rhodaminated EGF (1 µg/ml) or the 13A9 antibody (20 µg/ml in the presence of EGF) were added for 1 h at 0°C, followed by wash and shift at 37°C for 20–30 min to allow internalization. After fixation, 13A9 was detected with Cy3-conjugated secondary antibody. In assays with anti-hemagglutinin (anti-HA) (see Fig. 6B), 20 µg/ml anti-HA was substituted for 13A9. Quantitation was performed on at least two experiments, in duplicate (>100 cells per condition). Plasma membrane staining was compared with intracellular vesicle-associated staining. Internalizing cells displayed preponderant intracellular staining but little, if any, plasma membrane staining. Noninternalizing cells displayed the opposite phenotype.

Immunoelectron Microscopy. For preembedding immunolabeling (Figs. 1A and 2B Left and Center and Table 1), starved prechilled cells were incubated on ice for 30 min with 20 µg/ml anti-EGFR 13A9, then with rabbit anti-mouse (code no. Z0412, DAKO), and finally with 10-nm protein A-gold. Cells were then incubated at 37°C for 2 min with EGF (at the indicated doses), fixed for 15 min at room temperature (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2), and processed as described in ref. 10.

Abbreviations: KD, knockdown; Ub, ubiquitin; UBmut, mutant Ub; UIM, Ub-interacting motif; HA, hemagglutinin.

See Commentary on page 2679.

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ImmunoGold labeling on ultrathin cryosections (Figs. 2B Right and 4B) was performed as reported in ref. 10. Further details are in Supporting Materials and Methods.

**Results**

In HeLa cells, expressing endogenous EGFR, we found that EGFR follows different internalization routes, depending on the dose of EGF. We used two concentrations of EGF (low EGF, 1.5 ng/ml; high EGF, 20 ng/ml), both within the range of physiological EGF levels (1–2 ng/ml in serum; 10–100 ng/ml in several other biological fluids, see Supporting Materials and Methods). At low EGF, EGFR localized predominantly into clathrin-coated pits, whereas, at high EGF, the receptor was roughly equally partitioned between coated pits and caveolae (Fig. 1A). At low EGF, the receptor was significantly tyrosine phosphorylated and fully competent for downstream signaling (as demonstrated by activation of ERK), whereas significant ubiquitination was only detectable at high EGF (Fig. 1B). Thus, there is correlation between the state of EGFR ubiquitination and its partition into caveolae.

We have shown that a chimera encompassing the extracellular and transmembrane domain of the EGFR fused to Ub (EGFR/Ubmut) is constitutively internalized (4). By exploiting this chimera, whose internalization exclusively depends on the signaling abilities of the Ub moiety, we endeavored to determine how ubiquitination directs internalization. In EGF-negative NR6 (Fig. 2A) or Chinese hamster ovary (CHO) (data not shown) cells, the internalized EGFR/Ubmut colocalized extensively with caveolin-1, a marker of the non-clathrin lipid raft/caveolar pathway, but poorly with clathrin, whereas a transfected EGFR (EGFRwt) showed the opposite behavior (Fig. 2A). By immunoelectron microscopy, we detected EGFR/Ubmut exclusively in caveolae (20 cell profiles analyzed). (Bar, 83 nm.)

**Table 1. Morphometry from 10 cell profiles**

<table>
<thead>
<tr>
<th>Organelles</th>
<th>EGF at 1.5 ng/ml, % (n)</th>
<th>EGF at 20 ng/ml, % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled caveolae</td>
<td>4 (72)</td>
<td>22 (106)</td>
</tr>
<tr>
<td>Labeled coated pits</td>
<td>40 (35)</td>
<td>30 (46)</td>
</tr>
<tr>
<td>Gold in caveolae</td>
<td>1 (296)</td>
<td>12 (383)</td>
</tr>
<tr>
<td>Gold in coated pits</td>
<td>16 (296)</td>
<td>15 (383)</td>
</tr>
</tbody>
</table>

Shown is the percentage of labeled organelles, with values in parentheses representing the number of caveolae or coated pits counted, or the percentage of gold particles in organelles, with the values in parentheses representing the total gold particles detected on the plasma membrane.
In an initial set of experiments, we monitored internalization of rhodamine-conjugated EGF (Fig. 3 B and C; see Fig. 8, which is published as supporting information on the PNAS web site). DynaminII-K44A, a general endocytic inhibitor (12, 13), abrogated internalization of EGFR/Ubmut, Egfr, and a mutant EGFR (Y1045F), that cannot efficiently recruit Cbl (11), thus showing considerably reduced ubiquitination (Fig. 3A). All these constructs were expressed in EGFR-negative CHO or NR6 cells.

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A quantitative assessment was obtained by measuring the level of endocytosed EGF (Fig. 3D). At high EGF, although the internalization of EGFRwt was partially sensitive to filipin, the drug had no significant effect on endocytosis of the Y1045F mutant, but it reduced the internalization of EGFR/Ubmut to background levels (Fig. 3D).

To address this point, we knocked down (KD) eps15, eps15R (a related protein), and epsin in HeLa cells (Fig. 6A). The endocytosis of EGFR/Ubmut was abrogated in triple eps15/eps15R/epsin KD cells but not in individual KD cells (Fig. 6B; see Fig. 11A, which is published as supporting information on the PNAS web site). The reexpression of eps15 but not of an eps15-UIM-deleted mutant in the triple KD cells restored the internalization of EGFR/Ubmut (Fig. 6B, see Fig. 11B). Thus,
when a membrane protein is exclusively internalized as a function of its ubiquitination in a nystatin/filipin-sensitive pathway, the interaction with the UIM proteins eps15 and eps15R is essential. In addition, the functions of eps15 and eps15R are redundant in this pathway.

We then investigated the behavior of the endogenous EGFR. Initially, we investigated the role of clathrin because discordant results have been reported (8, 9, 23). We reasoned that the scarce effect of clathrin KD in one of these studies could be explained by the use of high EGF in the internalization assay (8). According to our present data, under those conditions the contribution of a clathrin-independent pathway (defined on the basis of its

![Image](https://example.com/image1.png)

**Fig. 4.** UIM proteins and EGFR/Ubmut. (A) φ-NX cells were transfected as indicated above the gels. Lysates (5 mg) were immunoprecipitated (IP) and immunoblotted (IB) as indicated. (B) Immunoelectron microscopy of NR6 expressing EGFR/Ubmut. (Bar, 83 nm.) Arrowheads point to caveolar membranes. Note that in cells expressing the EGFR/FLAG control chimera, eps15 and epsin were never detected in caveolae (data not shown). (C) CHO cells were cotransfected with EGFR/Ubmut and either GFP or GFP-UIM (amino acids 842–897 of eps15) and incubated with rhodamine-EGF (red, Left) or rhodamine-transferrin (red, Center). Merged images are shown. (Right) A quantitative assessment of EGF internalization (3 ng/ml [125I]-EGF for 15 min at 37°C).

![Image](https://example.com/image2.png)

**Fig. 5.** UIM proteins bind to ubiquitinated EGFR. (A) Lysates (5 mg) from HeLa cells were immunoprecipitated (IP) and immunoblotted (IB) with the indicated antibodies. (B) CHO cells were transiently cotransfected with either EGFRwt or Y1045F and individually transfected with epsin, eps15, and hepatocyte growth factor receptor substrate (Hrs). Lysates (2 mg) were immunoprecipitated/immunoblotted as indicated. (C) HeLa cells were transfected with the indicated FLAG-tagged constructs encoding wild-type eps15 or an eps15-UIM-deleted mutant [eps15859–897 (29)], or wild-type epsin, or an epsin-UIM-deleted mutant [epsin190–192 (29)]. FLAG is an empty control vector. Lysates (2 mg) were immunoprecipitated/immunoblotted as indicated.
nystatin/filipin sensitivity) would be substantial. As shown in Fig. 6C, at low EGF, the KD of clathrin (levels of clathrin in KD cells are in Fig. 6A) almost completely abrogated internalization, demonstrating that, under this condition, clathrin is absolutely required. Next, we tested whether UIM-containing proteins are required in this pathway. At low EGF, although the individual KDs of eps15/eps15R or epsin had no effect, the triple eps15/eps15R/epsin KD exhibited a significant decrease in EGFR internalization (Fig. 6C). Treatment with filipin had no effect in any of the KDs at low EGF (Fig. 6D). These results are in agreement with those reported by Huang et al. (23) and demonstrate that eps15/eps15R and epsin participate (redundantly) also in the clathrin pathway. However, they are not strictly essential, as clathrin endocytosis proceeds also in their absence.

At high EGF, clathrin KD reduced internalization by ∼50%. However, filipin treatment of clathrin KD cells reduced the residual internalization to background levels (Fig. 6D). These results show the existence of two modalities of internalization, one relying on clathrin (at low EGF) and one relying both on clathrin-dependent and clathrin-independent pathways (at high EGF). The triple eps15/eps15R/epsin KD also reduced EGFR endocytosis at high EGF, albeit in a filipin-insensitive fashion, demonstrating that, in the triple KD, the non-clathrin nystatin/filipin-sensitive component of EGFR endocytosis is completely abolished (Fig. 6D). Individual KDs of eps15/eps15R or epsin had no effect, reinforcing the notion of redundancy among these proteins (data not shown). Finally, the quadruple eps15/eps15R/epsin/clathrin KD reduced EGFR internalization at high and low doses to background levels (Fig. 6C and D). Together with data obtained with EGFR/Umut, these results prove that eps15/eps15R and epsin are (redundantly) indispensable to the non-clathrin internalization of the EGFR.

**Discussion**

Our studies unveil important differences in the mechanisms through which the EGFR couples with the machinery of internalization as a function of EGF dose. At low doses of ligand, the receptor is almost exclusively internalized through a clathrin-dependent mechanism, whereas, at higher doses, a non-clathrin pathway defined on the basis of its nystatin/filipin-sensitivity emerges and becomes as relevant as the clathrin pathway. Of note, low and high EGF concentrations are physiologically relevant, as discussed in Supporting Materials and Methods. The emergence of the non-clathrin pathway correlates with the appearance of detectable ubiquitinated EGFR. A molecular genetics approach provided a mechanistic basis for this correlation, because a mutant EGFR that can no longer be ubiquitinated (Y1045F) was internalized through the clathrin pathway, even at high doses of EGF.

The dependence of the non-clathrin pathway on ubiquitination was further confirmed by a reductionistic approach, which exploited chimeric molecules that can signal solely through an Ub moiety. It should also be pointed out that such molecules are not “true” representations of receptors. In the case of the EGFR-based chimera, we have previously demonstrated that the chimera is constitutively internalized and routed to the degradative compartment, even in the absence of ligand stimulation (4). Once, however, the relevance of ubiquitination in non-clathrin endocytosis was established on the holo-EGFR, as discussed above, the chimera proved very useful in simplifying the plethora of signals present on an activated receptor and in allowing studies of internalization solely as a function of cargo ubiquitination.

Our results are consistent with data showing that EGFR internalization through clathrin-coated pits requires Cbl but not receptor ubiquitination (5, 24). These latter findings also indirectly stress the importance of Cbl-executed ubiquitination of other downstream endocytic proteins for proper clathrin endocytosis. A recent report by Stang et al. (25), which shows that Cbl-dependent ubiquitination is required for progression of the EGFR into coated pits, further reinforces this notion. Taken
together with these results, our findings suggest a mechanism through which ubiquitination of endocytic machinery, but not cargo ubiquitination, is required for internalization into coated pits. At higher doses of EGF, however, when cargo ubiquitination becomes substantial, endocytosis is partially switched to a non-clathrin pathway.

How is this “switch” achieved? Our results directly implicate the recruitment of eps15/eps15R and epsin to the ubiquitinated cargo, as also shown by findings that expression of the EGFR/Ub chimera was able to induce relocalization of these proteins into caveolae. In addition, we showed that these proteins are redundantly essential in non-clathrin endocytosis of the EGFR. How the signal is further propagated downstream of these molecules in this pathway is not known and warrants further investigation. Furthermore, other Ub-binding proteins have been implicated in endocytosis. Thus, their potential role in the internalization of ubiquitinated cargo, possibly in a cell-specific fashion, also appears worth investigating.

Our results also indicate a role (albeit non-essential) for eps15/eps15R and epsin in clathrin internalization. Indeed, these proteins have been implicated in the formation of coated pits, and they have been proposed to function as adaptors between ubiquitinated cargo and components of the coat, such as AP2 and clathrin (2, 26). This latter possibility seems unlikely based on our present data, at least under a condition in which the clathrin route is paramount at low EGF concentrations, simply because there is not sufficient cargo ubiquitination at these doses. However, we have previously shown that clathrin-dependent synaptic vesicle recycling in Caenorhabditis elegans is negatively affected by the removal of eps15 through a mechanism that involves a genetic interaction with dynamin (27). It is possible, therefore, that, at least in the case of eps15, participation to clathrin endocytosis involves mechanisms different from interactions with ubiquitinated cargo.

Finally, we note that the TGF-β receptor internalizes through coated pits and caveolae (28). The two pathways were associated with increased receptor signaling and rapid receptor degradation, respectively (28). In the case of EGFR, at low EGF (when internalization proceeds exclusively through coated pits), the receptor is already fully competent for effector signaling (Fig. 1B). Conversely, at high EGF (when non-clathrin endocytosis becomes significant), there is no increase in signaling abilities but readily detectable increase in EGFR down-regulation (Fig. 1B). Thus, at minimum, caveolar/raft internalization does not apparently contribute to EGFR signaling, and possibly it is preferentially associated with receptor degradation.

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