Epidermal Growth Factor Induces Ubiquitination of Eps15*  
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Epidermal growth factor (EGF) receptor pathway sub-structure clone 15 (Eps15) has been described as a 142-kDa EGF receptor substrate. It has been shown to bind to the EGF receptor, adaptor protein-2, and clathrin and is present at clathrin-coated pits and vesicles. Upon stimulation of cells with EGF or transforming growth factor α, Eps15 becomes rapidly and transiently phosphorylated on tyrosine residues. This phosphorylation coincides with an increase of 8 kDa in molecular mass. Here we show that this increase in molecular mass is not due to tyrosine phosphorylation. Instead, we found both by Western blotting and protein sequencing that this EGF-induced increase in molecular mass is the result of monoubiquitination. Eps15 ubiquitination but not tyrosine phosphorylation was inhibited under conditions that blocked EGF-induced internalization of the EGF receptor. Our results establish ubiquitination as a second form of EGF-stimulated covalent modification of Eps15.

Eps15† has been identified as a 142-kDa substrate of the EGF receptor (1). In quiescent cells Eps15 is associated to the EGF receptor, and upon EGF stimulation this association increases dramatically (2). In addition, Eps15 has been shown to bind to both adaptor protein-2 and clathrin (2, 3). Subcellular fractionation and immunolocalization studies have shown that Eps15 is present in clathrin-coated pits and vesicles but not in early endosomes (2, 4). Eps15 shares homology with the yeast proteins End3p and Pan1p. Both proteins contain multiple Eps15 homology domains, a motif proposed to mediate protein-protein interactions. This indicates that EGF induces two different modes of post-translational modification of Eps15: tyrosine phosphorylation and ubiquitination.

Tyrosine kinase activity of the EGF receptor was found to be required for this apparent increase in molecular mass of Eps15 (7). Expression of Eps15 cDNA in bacteria shows the presence of only the 142-kDa form, suggesting that Eps15 is undergoing an EGF-induced post-translational modification (1). In this paper we investigated the nature of this post-translational modification of Eps15. We found that the appearance of the high molecular mass form of Eps15 is not due to EGF-induced hyperphosphorylation. Instead, we found that the 8-kDa increase in molecular mass was caused by monoubiquitination of Eps15. This indicates that EGF induces two different forms of post-translational modification of Eps15: tyrosine phosphorylation and ubiquitination.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture**—HER14 fibroblasts (NIH3T3 fibroblasts stably transfected with human EGF receptor cDNA) were cultured in bicarbonate buffered Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc., Gaithersburg, MD) supplemented with 7.5% (v/v) fetal calf serum (FCS) (Life Technologies, Inc.) in a humidified atmosphere at 37 °C.

**Immunoprecipitations**—Cells were grown in 60-, 100-, or 175-mm dishes (Nunc Life Technologies, Gaithersburg, MD) till 80% confluency. Cells were serum-starved in DMEM with 0% v/v FCS for 24 h before stimulation with 50 ng/ml EGF. Cells were lysed in RIP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 100 mM NaF, and 1 mM Na3VO4) at 4 °C for 5 min, scraped from the dish, and centrifuged for 5 min at 12,000 × g in an Eppendorf centrifuge. Total cell lysate samples were prepared by adding Laemmli sample buffer to the RIP lysates. For immunoprecipitations, the RIP lysates were incubated with 25 µl of a 1:1 suspension of protein A-Sepharose for 1 h at 4 °C and centrifuged. Supernatants were incubated with anti-Eps15 antibody (8) for 2 h at 4 °C. Subsequently, protein A-Sepharose was added, and after further incubation of 2 h, the immunoprecipitates were washed three times, once with RIP-buffer, once with high salt buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 1% Triton X-100), and finally once with low salt buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 1% Triton X-100). For alkaline phosphatase treatment, immunoprecipitates were incubated in phosphatase buffer (50 mM Tris, pH 8.5, and 1 mM EDTA) with 50 units of alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) at 37 °C for 30 min. Heat-inactivated alkaline phosphatase was prepared by incubating the alkaline phosphatase at 95 °C for 15 min. The beads were boiled in 20 µl of Laemmli sample buffer for 5 min, and proteins were separated by SDS-polyacrylamide gel electrophoresis, Western blotted onto PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA), and probed with rabbit polyclonal anti-ubiquitin antibodies (antibody kindly provided by Dr. A. Ciechanover). Protein bands were visualized by Enhanced Chemiluminescence (Amersham, Arlington Heights, IL) using peroxidase-conjugated goat-anti-rabbit or rabbit-anti-mouse immuno-globulins (Jackson Immunoresearch, Pennsylvania, PA).

**Edman Degradation**—To determine the N-terminal amino acid sequence of Eps15, proteins of immunoprecipitates were separated on a 8% SDS-polyacrylamide gel and transferred to a PVDF membrane. The proteins were stained with Ponceau S (Sigma, St. Louis, MO), and the Eps15 bands were cut out of the membrane, washed thoroughly with distilled water, and subjected to Edman degradation (9, 10).

**Inhibition of Endocytosis**—Inhibition of endocytosis in HER14 cells was performed by potassium depletion (11), by incubating the cells in hypotonic medium (12), by acidification of the cytosol (13), or by an incubation of the cells at 4 °C (14).

For potassium depletion, cells were washed twice with depletion buffer (20 mM Hepes, pH 7.4, 0.14 M NaCl, 1 mM CaCl2, 1 mM MgCl2, and 1 g/l p-glucose). Subsequently, cells were incubated for 5 min with
Supplemented with 10 mM KCl. Inhibition of endocytosis by hypertonic buffer at 37 °C. Control cells were incubated with the same buffer medium. For inhibition of endocytosis by acidification of the cytosol, with hypertonic medium before a 30-min incubation at 37 °C (Fig. 1).

These results demonstrate that the appearance of high molecular mass of Eps15 is not the result of tyrosine phosphorylation.

EGF Induces Ubiquitination of Eps15

To investigate the monoubiquitination of Eps15, Eps15 was immunoprecipitated from HER14 cells that were either left unstimulated or stimulated with 50 ng/ml EGF. The protein samples were separated on 8% SDS-polyacrylamide gels, and the Western blot was probed with anti-Eps15 antibodies. A clear mobility shift was seen after EGF stimulation but not in unstimulated cells (Fig. 2). Subsequently, the Western blot was stripped and reprobed with anti-ubiquitin antibodies. In this case only the 150-kDa form of Eps15 was detected, demonstrating that Eps15 becomes monoubiquitinated upon EGF stimulation (Fig. 2). In addition to the appearance of the 150-kDa band, a slight staining of higher molecular mass Eps15 was detected upon EGF addition. This phenomenon was better visible upon longer exposures (data not shown). Eps15 of higher molecular mass was previously also found on Western blots containing immunoprecipitated Eps15 that were stained for phosphotyrosine residues (2). These observations suggest that Eps15 is not only monoubiquitinated but that a minority of Eps15 may also be mult ubiquitinated.

The 150-kDa Form of Eps15 Contains Covalently Bound Ubiquitin—To obtain further proof for the ubiquitination of Eps15, the N-terminal sequences of the 142- and 150-kDa Eps15 isoforms were determined by Edman degradation. Because ubiquitin is conjugated via its C terminus to the target proteins, the N terminus of conjugated ubiquitin is still available for Edman degradation. Sequencing of the 142-kDa form of Eps15 did not result in any signal, most probably due to N-terminal blocking. Sequencing of the 150-kDa form of Eps15 resulted in a single protein sequence (Fig. 3). Comparison of these 10 amino acids with the published sequence of bovine ubiquitin revealed that the obtained amino acids are identical to the first amino acids of ubiquitin. Comparison of this sequence with sequences in the SWISS-PROT protein data base did not reveal a relevant match with any other protein than ubiquitin.

Based on both the Western blotting results and the N-terminal amino acid sequence, we conclude that Eps15 becomes ubiquitinated after stimulation of the cell with EGF. Because the increase in molecular mass of Eps15 is similar to the
molecular mass of ubiquitin (8 kDa), we conclude that Eps15 becomes predominantly monoubiquitinated. Because the approximate ratio of the two Eps15 forms in EGF-stimulated cells was previously determined as 1:1, we estimate that about 50% of Eps15 becomes monoubiquitinated after stimulation of cells with EGF (2). Both forms of Eps15 become phosphorylated on tyrosine residues (Fig. 1), which indicates that ubiquitination of Eps15 is not required for its phosphorylation.

Monoubiquitination of proteins has not frequently been reported. Examples of monoubiquitination are the T cell antigen receptor (17), histone H2A (18), and cytochrome c (19). The yeast α-factor receptor has recently been shown to become either mono- or diubiquitinated (20). Multisubunit ubiquitination of proteins usually starts on one lysine residue (16). Subsequently, this ubiquitin becomes ubiquitinated, resulting in the formation of multiquitin chains. Examples of multisubunit ubiquitination include cytoplasmic and nuclear proteins but also integral membrane proteins such as receptors for EGF (21), growth hormone (22, 23), platelet-derived growth factor (24), and the tumor necrosis factor (25). Protein ubiquitination has been implicated in many cellular processes (16). The most widely studied function of ubiquitination lies in the targeting of (multiquitinated) proteins for degradation to the 26 S proteasome. However, not all ubiquitinated proteins are degraded, suggesting additional functions for ubiquitination besides protein synthesis. Treatment of cells with transcription inhibitors resulted in a reduced level of ubiquitinated histone H2B, suggesting a role for ubiquitination in chromatin organization (18). Recently, it has been suggested that ubiquitination plays a role in the activation of IκBα, a regulator of the transcription factor NFκB (26). Interestingly, a new function for ubiquitination has been recently described for the ubiquitination of plasma membrane receptors. Ubiquitination of both the growth hormone receptor and the α-factor receptor in S. cerevisiae have been implicated in the endocytosis of these receptors (20, 23, 27).

The binding of Eps15 to both adaptor protein complex-2 and clathrin and the presence of Eps15 in clathrin-coated pits and vesicles of mammalian cells suggest a role for Eps15 in the endocytosis of the EGF receptor. To investigate the possible relationship between Eps15 ubiquitination and EGF receptor internalization, we examined the effect of blocking EGF receptor internalization on Eps15 ubiquitination. Internalization of EGF receptors was inhibited in four different ways: by incubation at low temperature, by depleting potassium from the cytosol, by hypertonic shock, or by acidification of the cytosol. These methods inhibit different steps in endocytosis: hypertonic shock and incubation at low temperature inhibit endocytosis of the EGF receptor internalization (11), whereas acidification of the cytosol is suggested to inhibit pinching off of clathrin-coated pits from the plasma membrane (13). The effect of these conditions on EGF endocytosis was measured using 125I-labeled EGF. In control cells EGF was rapidly internalized, whereas under all four endocytosis inhibiting conditions EGF internalization was inhibited for more than 80% (Fig. 4). Analysis of Eps15 phosphorylation revealed that in all cases Eps15 became phosphorylated on tyrosine residues (Fig. 5B). These results demonstrate that EGF receptor activity has not been affected by either of these treatments. Interestingly, it was recently reported by Vieira and co-workers (28) that inhibition of endocytosis using a dynamin mutant resulted in differences in EGF receptor activity. Together these data show that when endocytosis is inhibited Eps15 monoubiquitination is abolished, but tyrosine phosphorylation remains undisturbed.

An interesting question is the possible function of the monoubiquitination of Eps15. The absence of Eps15 ubiquitination under conditions that inhibit EGF receptor internalization indicates that either Eps15 ubiquitination is required for Eps15 endocytosis or EGF receptor endocytosis is a prerequisite.
site for Eps15 ubiquitination. The first possibility is an analogy to what has been reported for the growth hormone receptor in mammalian cells (23) and the α-factor receptor in yeast (20). In this case Eps15 ubiquitination could be involved in the early steps of endocytosis of the EGF receptor. However, inhibition of endocytosis of the α-factor receptor in yeast resulted in an increased ubiquitination of the receptor, which is in contrast to the results presented in this paper (20). Alternatively, our results may indicate that endocytosis is required for Eps15 ubiquitination. This would imply that Eps15 ubiquitination occurs exclusively at a post-surface endocytic transport step. We have shown previously that Eps15 localization is restricted to coated pits and coated vesicles and absent from early endosomes (2). The monoubiquitination of Eps15 could thus be involved in the targeting of coated pits to the early endosome or in the uncoating of the coated vesicle. Another possibility is that Eps15 ubiquitination could be involved in the targeting of Eps15 to the 26 S proteasome for its degradation, which is in fact the first described function of protein ubiquitination.

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