Internalization and Down-regulation of the Human Epidermal Growth Factor Receptor Are Regulated by the Carboxyl-terminal Tyrosines*

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The C terminus of the epidermal growth factor receptor (EGF-R) contains three tyrosines (Y1068, Y1148, and Y1173) which correspond to the major autophosphorylation sites. To investigate the role of the tyrosines in internalization and down-regulation of the EGF-R, mutational analysis was performed with receptors in which 1, 2, or all 3 tyrosines were changed to phenyalanines. The triple point mutant EGF-R, expressed in NIH-3T3, exhibited low autophosphorylation in vivo, low biological and reduced kinase activities. Single and double point mutants were down-regulated, as well as wild type EGF-R in response to EGF showing a half-life of about 1 h. Degradation of the triple point mutant, however, was impaired and resulted in a half-life of 4 h in the presence of EGF. EGF-dependent down-regulation of surface receptors was decreased in the triple point mutant EGF-R as was internalization and degradation of EGF. The specific rate of internalization of the triple point mutant was reduced. By contrast, intracellular processing of ligand previously internalized at 20 °C was similar between wild type and mutant receptors. Taken together the data indicate that the delay in degradation observed in cells expressing the triple point mutant EGF-R can be attributed mainly to a slower removal from the cell surface. Our results show that in the full-length EGF-R all three C-terminal tyrosines are necessary for rapid internalization, suggesting that autophosphorylation is required for efficient EGF-dependent receptor endocytosis.

The EGF receptor (EGF-R) is a cell surface glycoprotein able to bind EGF and transforming growth factor α, thereby mediating their mitogenic effects (1, 2). Binding of EGF results in activation of rapid events including autophosphorylation, changes in membrane potential, phosphatidylinositol turnover, elevation of cytoplasmic Ca\(^{2+}\) and intracellular pH, and phosphorylation of cellular substrates (2-4).

Another very early event triggered by binding of EGF is down-regulation of both ligand and receptor (5, 6). Prior to activation the EGF-R is diffusely distributed on the plasma membrane. Upon EGF addition the receptor accumulates in coated pits, is rapidly internalized in endocytic vesicles and delivered to lysosomes for degradation (7, 8). In the presence of EGF the half-life of the EGF-R is reduced to approximately 1 h in most cultured cells (7-9), with little evidence of recycling (10, 11). In hepatocytes in vivo there is evidence that receptors recycle several times before degradation (12-15).

For recycling receptors which are selectively localized in coated pits such as receptors for low density lipoprotein and transferrin, a consensus sequence important for internalization has been identified (16-19). These receptors, however, permanently reside in coated pits, while growth factor receptors, like the EGF-R, only accumulate in the pits upon ligand binding to undergo endocytosis (20, 21).

Although recent deletion analysis delimits a region essential for EGF-dependent receptor internalization (22), the molecular signals inducing internalization and degradation upon EGF binding are still poorly understood. There is substantial experimental evidence that points to a central role of phosphorylation in inducing receptor down-regulation. The EGF-R intrinsic tyrosine kinase activity is essential for receptor degradation (23, 24); however, while one group has reported that the kinase-negative receptor is able to internalize in response to EGF (24, 25), another finds no internalization at all (22, 23). In addition, the second group finds that microinjection of antiphosphotyrosine antibodies prevents EGF-induced endocytosis (26). Phosphorylation of EGF-R by other cellular kinases seems also to affect the normal pathway of receptor internalization. In the absence of EGF or transforming growth factor-α, activation of protein kinase C by phorbol esters induces phosphorylation of the EGF-R on Thr-654 (27, 28) and its internalization without degradation (29, 30). Furthermore, it has recently been reported that mutations of 2 amino acids of the EGF-R, threonine 669 and serine 671, which can be phosphorylated by a cellular kinase different from protein kinase C, also affect receptor internalization (31).

To directly test the role of autophosphorylation on EGF-dependent down-regulation, we have used EGF-R mutants with point mutations in the three C-terminal autophosphorylation sites. The biological activity of the point mutants is gradually reduced in parallel with the substitution of the tyrosine residues. In this paper we report that mutation of all three autophosphorylation sites which results in a mutant with low autophosphorylation, reduced kinase activity, and low biological activity, drastically reduces EGF-dependent receptor internalization. These results indicate that in the full-length receptor all 3 C-terminal tyrosines are necessary to mediate rapid receptor internalization and suggest that

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The abbreviations used are: EGF-R, epidermal growth factor receptor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium.

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8363
phosphorylation of these sites may expose a domain required for internalization.

**EXPERIMENTAL PROCEDURES**

**Materials—**
$\text{EGF (100-180 µCi/µg) was from Amersham Corp.}$

$\text{[3H]methionine (1100 Ci/µmol) were from Du Pont-New England Nuclear.}$

$\text{Mouse EGF was from Bethesda Research Laboratories, fibronectin from Upstate Biotech-}$

$\text{nicontin, and transferrin from Sigma Chemical Co.}$

$\text{The method of D.} \text{P.} \text{D.}$

$\text{Di Fiore and D.}$

$\text{Bottaro (National Institutes of Health) (33).}$

**Mutant Construction Transfection and Cell Culture—**

$\text{The EGF-R mutants were made by oligonucleotide site-directed mutagenesis of}$

$\text{Tyr-1173, -1148, and -1068 into Phe as previously described.}$

$\text{Affinity purified polyclonal antiphosphotyrosine antibody, a-Pyr 4387, was kindly provided by}$

$\text{Drs. P.}$

$\text{P.}$

$\text{D.}$

$\text{Di Fiore and D.}$

$\text{Bottaro (National Institutes of Health) (33).}$

**RESULTS AND DISCUSSION**

In Vivo Autophosphorylation of Wild Type and Mutant EGF Receptors—To clarify the role played by autophosphorylation in EGF-R internalization and down-regulation, we have used EGF-R mutants in which the three main C-terminal autophosphorylation sites, Y1173, Y1148, and Y1068, were mutated into alanine as previously described.

To study the role of mutagenesis of the EGF-R, monolayers were incubated with 10 nM [125I-EGF for 1 h at 37 °C followed by 10 min at 37 °C to allow internalization but not degradation (25, 40). Surface-bound EGF was then removed by an acid wash, cells were washed as described above, incubated at 37 °C for different times with 100 nM unlabeled EGF, and then processed as above.

In Vivo Autophosphorylation—Cells were plated on fibronectin-treated dishes and when confluent starved overnight in DMEM supplemented with 100 µg/ml of ferric ammonium citrate (FAC) and 37 °C. The cells were treated with or without 50 nM EGF for 5 min and immediately lysed in 1% Triton X-100 containing 2 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 5 mM sodium orthovanadate (Na3VO4), and 10 mM sodium pyrophosphate. Total proteins were determined by the method of Bradford (44) (Bio-Rad). Equal amounts of proteins (100 µg) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel (37), electrotransferred to nitrocellulose, and exposed to either anti-EGF-R antibody Ab2913 or antiphosphotyrosine antibody a-Pyr 4387 and [3H]methionine incorporation in cell lysates by trichloroacetic acid was performed as described above. Results were similar with cells expressing low and high numbers of EGF-R.

**In Vivo Kinase Activity—**

NIH-3T3 cells expressing wild type and mutant receptors were stimulated with EGF, cell extracts blotted on nitrocellulose filters, and the phosphorylated receptor revealed by an antiphosphotyrosine antibody and shown to be similar for all the mutants (Fig. 1, panel A).

**In Vitro Autophosphorylation**—Cells were plated in 24-well plates and incubated as described above.

Monolayers were washed twice with ice-cold serum-free DMEM and then with binding medium containing 1 nM of [125I-EGF. Medium was then removed and cells were rapidly warmed and incubated at 37 °C for the indicated time. Cells were then washed four times with ice-cold binding medium. Surface-associated EGF was determined by acid-stripping (39) and internalized EGF was determined by measuring the released radioactivity after solubilization in 1 ml of 1 M NaOH. The amount of degraded (trichloroacetic acid soluble) and intact EGF (precipitable by trichloroacetic acid) was determined by precipitating the incubation medium with 10 mM trichloroacetic acid and counting supernatant and pellet. As control, the proportion of trichloroacetic acid-precipitable counts in the native [125I-EGF was determined to be 90-95%.

To study the role of mutagenesis of the EGF-R, monolayers were incubated with 10 nM [125I-EGF for 1 h at 37 °C followed by 10 min at 37 °C to allow internalization but not degradation (25, 40). Surface-bound EGF was then removed by an acid wash, cells were washed as described above, incubated at 37 °C for different times with 100 nM unlabeled EGF, and then processed as above.

**In Vivo Internalization and Degradation**—Cells were plated in 24-well plates and incubated as described above. This finding is in agreement with the lower kinase activity of the point mutants observed in vitro toward exogenous substrates and the low activity in vivo in mediating autophosphorylation.
Autophosphorylation and EGF-R Internalization

FIG. 1. In vivo tyrosine kinase activity of wild type and mutant EGF receptors. NIH-3T3 cells expressing wild type (lanes 1) and mutant EGF-R, MI 31 (lanes 2), MI 33 (lanes 3), and MI 34 (lanes 4) were treated without (-) or with (+) 50 nM EGF for 5 min at 37 °C prior to lysis. One hundred micromgrams of total protein were transferred to a nitrocellulose filter. Lysates were separated on a 7% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. A, phosphotyrosine containing EGF-R were identified with a polyclonal α-Ptyr antibody coupled to 125I-EGF-R antibody coupled to 35S-protein A. Sizes are shown in kilodaltons. Autoradiography was for 38 h for the α-Ptyr (A) and 16 h for the α-EGF-R blot (B). Autoradiograms exposed in the linear range were scanned and quantified. The content of phosphotyrosine incorporated in the EGF-stimulated EGF-R bands (Fig. 1) was then normalized for the total amount of EGF-R present in each lysate (B). MI 31 (YYF) showed a 2.5-fold decrease, and MI 33 (YFF) and MI 34 (FFF) EGF-R a 5-fold decrease in relative phosphotyrosine content compared to wild type EGF-R.

FIG. 2. EGF-dependent degradation of [35S]methionine-labeled wild type (WT) and mutant EGF receptors. A, cells expressing wild type and EGF-R mutant MI 31 (YYF), MI 33 (YFF), MI 34 (FFF), and Dc123 were labeled overnight with [35S]methionine, washed, and incubated in complete medium with 50 nM EGF for 15 (lane 2), 30 (lane 3), 60 (lane 4), and 120 min (lane 5) at 37 °C, or immediately lysed (lane 1). EGF-Rs were immunoprecipitated with a polyclonal α-EGF-R antibody, separated by 7.5% SDS-polyacrylamide gel, and subjected to autoradiography as described under "Experimental Procedures." B, the relative number of residual receptors for each mutant is plotted as a function of incubation time with EGF. Quantitation was performed both by direct scanning of autoradiograms exposed in the linear range and by scintillation counting of the solubilized [35S]-labeled receptor's band.

EGF-dependent phosphatidylinositol hydrolysis and cytoplasmic [Ca2+] elevation.3 EGF-dependent Half-life of Wild Type and Mutant EGF Receptors—To determine if EGF-dependent degradation of EGF-R was affected by the tyrosine substitutions, mutant cells were metabolically labeled with [35S]methionine and EGF was added for different times. The EGF-R was immunoprecipitated and the amount of residual receptor determined (Fig. 2). In the absence of EGF, no difference in the stability of mutant receptors was observed, the half-life being approximately 10–12 h in NIH-3T3 cells (Table I). In the presence of EGF, similar half-lives (approximately 1 h) were observed for wild type and point mutants with 1 (1173F and 1068F) or 2 tyrosine substitutions (1173F-1148F). These results are in agreement with our previous finding with the corresponding deletion mutants (9) and with the data on single mutants by Honegger et al. (42). In contrast, degradation of the triple point mutant was significantly delayed in the presence of EGF, resulting in a prolonged half-life (4 h compared to 1 h). (Fig. 2 and Table I). This result suggests that phosphorylation of the receptor on all 3 tyrosines is important for the rapid disappearance of the receptor. A small but consistent change was also observed with the comparable deletion mutant Dc123, which lacks all four autophosphorylation sites together with the last 125 amino acids, whose half-life was 1.5 h. In agreement with this finding, Chen et al. (22) have reported that a similar deletion mutant is removed slightly less efficiently from the surface when compared with wild type. The relative efficient degradation of the deletion mutant compared to the triple point mutant suggests either that conformational changes in the C terminus of the truncated receptor or compensatory autophosphorylation sites allow sufficient processing of the deletion mutant.

EGF-R Down-regulation of Wild Type and Triple Point Mutant EGF-R—Several possibilities could account for the prolonged half-life of the triple point mutant EGF-R. The initial internalization step might be affected so that mutant receptors were removed from the membrane more slowly; alternatively, lysosomal delivery might be impaired so that receptors would recycle several times or accumulate in an intracellular compartment. To investigate whether endocytosis was impaired in the mutant receptor, three types of internalization experiments were performed. As shown in Fig. 3 cells expressing wild type or triple point mutant receptor (MI 34) were preincubated with excess EGF for different times and the residual amount of receptor present

| Mutant | +EGF | -EGF |
|--------|------|------|
| Wild type (YYY) | 55 | 12 |
| MI31 (YYF) | 60 | 12 |
| MI37 (FYF) | 60 | ND |
| MI33 (YFF) | 50 | 9 |
| MI34 (FFF) | 240 | 11 |
| Dc123 | 85 | 12 |

*Cells were incubated with 50 nM EGF for 15, 30, 60, 120, and 240 min at 37 °C. The half-life was calculated from two (MI 37, MI 33, Dc 120) or four independent experiments (wild type, MI 31, MI 34).

†ND, not determined.
was still at the cell surface (Fig. 4, panel B). The slower internalization was reflected in a more persistent accumulation of ligand inside the cells. After 2 h, more than 45% of the radioactivity was still cell-associated and less than 40% was found degraded in the medium.

Ligand dissociation was similar for wild type and triple mutant EGF-R under these conditions and could not explain the difference in ligand removal from the cell surface between wild type and triple point mutant. After 10 min, dissociation accounted for 30 and 37% of total counts, respectively, in wild type and MI 34, after 30 min, 42 and 50% of total.

In order to directly localize the receptor, a time course similar to that shown in Fig. 4 was performed by immunofluorescence using a monoclonal anti-EGF-R antibody (EGF-R1). After 1 or 2 h of EGF treatment, the triple mutant EGF-R showed a more heterogeneous pattern of localization, diffuse cell surface fluorescence, as well as localization in peripheral endocytic vesicles with little staining in vesicles in the perinuclear region. In contrast, after 2 h little fluorescence was observed in cells expressing the wild type receptor and all localized in lysosomes, in agreement with the short half-life of the normal receptor in the presence of EGF.

To better quantitate the initial delay in internalization of the triple point mutant receptor, cells were incubated with 125I-EGF at 37 °C and surface bound versus internalized EGF was measured in the first 10 min (Fig. 5). Calculation of the internalization rate constant (41) revealed a 2-fold reduction for the mutant receptor compared to wild type (0.038 min⁻¹ versus 0.075 min⁻¹). The reduced internalization rate of MI 34 mutant was observed both at lower (1 nM, Fig. 5) and higher concentrations of EGF (5 nM, data not shown), suggesting that it is an intrinsic property of the mutant receptor, independent of the proportion of occupied receptors.

Taken together these data show that the triple point mutant was internalized more slowly and this effect resulted in a prolonged half-life in the presence of the ligand. This suggests that phosphorylation at the proper sites in the full-length receptor is important for rapid internalization leading to rapid degradation.

Intracellular Processing—We also tested whether intracellular processing of the mutant receptor was affected by the substitution of the C-terminal tyrosines. Cells were preincubated with 125I-EGF for 1 h at 20 °C to induce internalization but not degradation (40), surface-bound EGF removed by an

\[ K. \text{ Helin and L. Beguinot, unpublished observations.} \]

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**Fig. 4. Internalization and degradation of EGF by wild type and MI 34 (FFF) EGF receptors.** Wild type (A) and MI 34 (B) cells were kept on ice for 2 h with 1 nM 125I-EGF. Medium was removed, cells were rapidly warmed to 37 °C and incubated for the indicated time. EGF bound to the surface (■) was determined by an acid wash, internalized EGF (○) was determined by solubilizing the cells with 1.0 M NaOH and degraded EGF (□) in the supernatant was measured as trichloroacetic acid-soluble counts. Initial counts bound on the surface were 5250 and 3850 cpm for the wild type and MI 34 cells, respectively, using cells expressing high numbers of receptors. Each point is the average of triplicate measurements. Values are expressed as percentage of initial bound counts at time 0. The results have been confirmed twice with cells expressing both high and low numbers of EGF receptors.

**Fig. 5. Specific internalization rate of occupied wild type (WT) and MI 34 (FFF) EGF receptors.** Wild type (●) and MI 34 (○) cells pre-equilibrated at 37 °C were incubated with 1 nM 125I-EGF at 37 °C. At 2-min intervals plates were rapidly rinsed at 0 °C, and surface-associated and internalized 125I-EGF was determined as described under "Experimental Procedures." Each point is the average of triplicate determinations. The data are presented as a ratio of internalized EGF/surface EGF versus time. Internalization rate constant for the wild type EGF-R was 0.075 min⁻¹ and 0.038 min⁻¹ for the FFF receptor. Identical internalization constants were obtained with 5 nM 125I-EGF and with cells expressing both low and high numbers of EGF receptors.
acid wash, and cells warmed to 37 °C in the presence of 100 nM unlabeled EGF. Removal of surface EGF and addition of excess cold EGF were used to directly follow only the previously internalized and to avoid rebinding of 125I-EGF. As shown in Fig. 6, in cells expressing the mutant receptors, the total amount of internalized 125I-EGF was 3-fold less than for wild type cells. This is in agreement with the previous experiments (quantitation is given in the legend for Fig. 6) and indicates that at 20 °C the internalization of the mutant receptor was also impaired. However, the kinetics of disappearance of cell-associated radioactivity and of reappearance of EGF in the medium, as well as chloroacetic acid-soluble and -precipitable counts, were similar in both wild type and mutant cells. This finding suggests that once internalized, EGF was processed similarly and that under these conditions apparently intact EGF was released from both types of cells.

The EGF released in the medium comigrated with native 125I-EGF on SDS gel (data not shown). Whether these chloroacetic acid-precipitable counts represent biologically active or a partially inactive form of EGF (11) is not clear at present since it has proven difficult to perform binding studies with such a low amount of 125I-EGF. While it is possible to conclude a similar processing of internalized ligand and to suggest a similar processing for wild type and mutant receptors, we cannot directly demonstrate recycling of EGF and of receptors.

In the presence of monensin, a 20–30% decrease of surface-bound EGF and a similar increase of intracellular EGF was observed both in wild type and mutant cells. This result also indicates that the behavior of wild type and mutant EGF-R were similar and suggests that some recycling was occurring for both types of receptors. While Felder et al. (25) showed a difference in intracellular processing between wild type and kinase-negative EGF receptors, indicating that in response to EGF only the kinase-negative mutant was recycling, we find no significant difference in the intracellular processing between the wild type and the triple point mutant receptors. Our results complement the findings of Chen et al. (23) and Honegger et al. (24) that EGF-R kinase activity is essential for receptor degradation and the additional observation of Chen et al. (22) that a kinase-negative EGF-R is unable to undergo ligand-induced internalization.

A likely possibility is that EGF-dependent phosphorylation of the C-terminal tyrosines, induced by activation of the kinase, allows exposure of an internalization domain which is apparently required for rapid receptor internalization (22). Deletion mutants lacking more than this extreme C terminus, as the one used by Chen et al. (22), might not require this regulatory function and could be in a conformation which allows internalization. Indeed, in the 123 amino acids C-terminal deletion, Dc123, which lacks all four autophosphorylation sites, EGF-induced internalization and degradation occurred quite efficiently (Fig. 2), suggesting that the internalization domain may be sufficiently exposed to serve its role. Alternatively, since the deletion mutant bears at least one compensatory site for autophosphorylation, phosphorylation on Tyr-992 (43) (or on an as yet unidentified Tyr) could be a sufficient signal for this purpose. Phosphorylation of this residue does not appear to take place in the triple point mutant. Interestingly Tyr-992 is located just downstream of the putative internalization domain (22).

It is also possible that kinase activity is essential since rapid EGF-R internalization requires phosphorylation of substrates. The triple point mutant has a lower kinase activity in vitro and in vivo toward substrates (5, 6) and this low activity might account for the reduced internalization. Regardless of the molecular mechanism, however, we can conclude that in the full-length protein the C-terminal tyrosines are important for rapid internalization.

In conclusion, the C-terminal tyrosines seem to play a dual role in the regulation of the EGF-R receptor activity. We have recently reported that they are important to positively modulate the overall biological activity of the intact EGF-R and to activate its kinase function. Activation of EGF-R via autophosphorylation also serves as a signal for down-regulation of the mitogenic signal, triggering the removal of receptors from the surface and thereby creating a delicate control mechanism for EGF-R action.

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Note Added in Proof—Independent research by A. Sorkin, C. Waters, K. A. Overholsen and G. Carpenter, also described in this issue, have reached very similar conclusions concerning behavior of these EGF-R mutants.

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