Epidermal Growth Factor Receptor Interaction with Clathrin Adaptors Is Mediated by the Tyr\textsuperscript{974}-containing Internalization Motif*

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The carboxyl-terminal regulatory domain of the epidermal growth factor (EGF) receptor is essential for its endocytosis and interaction with the clathrin-associated protein complex AP-2. To identify AP-2 binding motif(s) in the receptor, several single and multiple-point mutations within the region between residues 966 and 977 of the human EGF receptor were made, and the mutant receptors were expressed in NIH3T3 cells. Mutation of tyrosine 974 alone or together with surrounding residues and the deletion of residues 973–975 essentially eliminated AP-2 co-immunoprecipitation with the EGF receptor. Furthermore, a synthetic peptide corresponding to receptor residues 964–978 blocked AP-2 association with the wild-type EGF receptor. These data suggest that AP-2 has only one high-affinity binding site in the EGF receptor composed of Tyr\textsuperscript{974}-containing motif. Receptor mutants that did not bind AP-2 displayed a lower rate of internalization, down-regulation, and turnover compared to wild-type receptors when expressed at high levels. However, similar receptor mutants expressed at low levels were internalized and down-regulated as efficiently as wild-type receptors. Internalization of the mutant receptors lacking the high-affinity binding site for AP-2 was inhibited by K\textsuperscript+-depletion of the cells, indicating that their endocytosis required intact coated pits. We suggest that whereas one mechanism of EGF receptor recruitment into coated pits involves high-affinity binding of AP-2 to Tyr\textsuperscript{974}-containing motif, another pathway may be mediated by weak receptor/AP-2 interactions or by proteins other than AP-2.

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¶ The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; AP-2, clathrin adaptor protein complex.

** Analysis of the kinetics of EGF endocytosis revealed that the specific rate of internalization is maximal when a low number of receptors are occupied by EGF and decreases with the increase of receptor occupancy (9, 10). The saturability of the endocytic pathway suggested that the mechanisms of EGF-induced receptor endocytosis are somewhat different from those regulating non-saturable endocytosis of cargo receptors, such as receptors for transferrin and low density lipoprotein. For instance, it has been found that tyrosine kinase activity and autophosphorylation of the receptor are necessary for EGF internalization via saturable pathway (11–15). However, similar to endocytosis of transferrin and low density lipoprotein receptors, internalization of EGF receptors requires the presence of "endocytic codes" in its intracellular domain. Three regions of the receptor carboxyl terminus have been identified, each was sufficient per se to promote rapid EGF internalization (16, 17). These regions contain sequences analogous to the endocytic codes of other receptors (17, 18). Two of these sequence motifs support rapid endocytosis when substituted for the internalization motif of the transferrin receptor (17). However, because internalization sequences of the EGF receptor were identified using large deletions of the receptor molecule, it is not clear whether all these codons function in the context of the native full-length EGF receptor.

Presence of endocytic codes in the intracellular tail of the receptor correlates with coated pit localization and efficient internalization of the receptor. It is proposed that internalization sequences directly interact with clathrin adaptor protein complex AP-2 and that this interaction represents the mechanism of the selective receptor recognition by coated pits (19, 20). AP-2 is the major component of coated pits and vesicles derived from the plasma membrane. It is a heterotetramer containing two large (~ 100 kDa) subunits (also called adaptins), \(\alpha\) (\(\alpha\) or \(\alpha\)C) and \(\beta\), a medium (50 kDa) and a small subunit (17 kDa) (reviewed in Ref. 21). Although functions of the individual subunits are not yet precisely determined, recent report by Ohno et al. (22) suggested that the medium subunit serves for specific interaction of AP-2 with tyrosine-containing endocytic sequences.

The hypothesis of the direct binding of receptors to AP-2 is based on experiments in which this interaction was demonstrated in vitro using purified components (19, 22–25). AP-2
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Materials—EGF was obtained from Collaborative Research Inc. 125I-EGF was prepared as described previously (33). Polyclonal rabbit 986 and 451 antibody to EGF receptor (2) were a gift from Dr. G. Carpenter (Vanderbilt University). Anti-EGF receptor rabbit serum 2913 (specific to intracellular domain) was described elsewhere (1). Monodonal antibodies AC1-M11 that recognize α-subunits of AP-2 were a gift from Dr. M. S. Robinson (University of Cambridge, United Kingdom). Polyclonal antibodies 31 (Ab31) to α-C-subunit were developed and characterized in our previous studies (27). Polyclonal antibodies were used as the IgG-fraction purified from the serum using Protein A-Sepharose (Sigma).

Immuno precipitation of EGF Receptors and AP-2—Cells were grown on 150-mm culture dishes and used when confluent. Cells were treated or not with EGF (100 ng/ml) in binding medium (DMEM, 0.1% bovine serum albumin, 20 μM Hepes, pH 7.3), washed with Ca2+/Mg2+-free phosphate-buffered saline and solubilized by scraping with a rubber policeman in TGH buffer (1% Triton X-100, 10% glycerol, 50 mM NaCl, 5 mM Hepes, pH 7.3. All solutions were made with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 544 μM iodoacetamide, 10 μg/ml aprotinin) in the absence or presence of sodium deoxycholate (1%) followed by gentle rotation for 10 min at 4°C. Lysates were then centrifuged at 100,000 × g for 20 min. Approximately 50 or 95-99% of the total cellular AP-2 pool was found in the supernatants after centrifugation of TGH or TGH-sodium deoxycholate extracts, respectively. Supernatants were incubated with anti-EGFR (986, 451) or Ab31 for 3–15 h at 4°C and then 30–60 min after the addition of Protein A-Sepharose (Sigma). Preimmune rabbit serum or rabbit IgG (2ymed Inc.) were used for nonspecific control. AP-precipitations were also controlled for nonspecific immunoreactivity by immunoprecipitations with the specific anti-AP IgG in the presence of an excess of the respective antigen.

Immunoprecipitates were washed twice with TGH or TGH-sodium deoxycholate supplemented with 100 μM NaCl and then once without NaCl. 7.5% SDS-polycrylamide gels were used to separate proteins. Transfer to nitrocellulose membrane and protein immunoblotting were carried out as described (27). The top (above the 116-kDa molecular mass marker) and bottom portions of the nitrocellulose membrane were blotted, respectively, with the mixture of antibodies to the EGF receptor and adaptor antibody (AC1-M11 to both α-subunits or Ab31 to C-subunit). Sheep antibodies to mouse IgG (Cappel Inc.) or protein A (2ymed Inc.) conjugated with horseradish peroxidase and enhanced chemiluminescence (Amersham or DuPont NEN) were used to detect the proteins, respectively. A Bio-Rad PhosphorImager was used for quantitation.

Peptide Competition Experiments—Peptide corresponding to amino acid residues 964–978 of the EGF receptor was synthesized and purified by Peptide Technologies Corp. WT-3 cells grown on 250-mm dishes were washed with Ca2+/Mg2+-free phosphate-buffered saline and solubilized in TGH as described above. Aliquots of lysate were incubated with 125I-EGF (0.1–1.0 nM) for 1 min at 4°C and then sodium deoxycholate (1%) was added to the lysates to induce AP-2 binding to EGF receptors. EGF receptors and AP-2 were then immunoprecipitated with anti-EGFR or Ab31, respectively. EGF receptors and α-adaptins were detected, correspondingly, in AP-2 and EGF receptor immunoprecipitates as described above. Alternatively, receptor or AP-2 immunoprecipitates were incubated in the presence of 200 μg of TGH of TGH-sodium deoxycholate containing peptide 964–978 (up to 1 μM) for 1 h at 4°C, washed in TGH-sodium deoxycholate followed by detection of co-precipitated receptors or α-adaptins by immunoblotting as described before. Peptide corresponding to the amino acid sequence of EGF receptor between 1168 and 1179 (containing Tyr1175) (40) was used as a control for the specificity of peptide 964–978.

Internalization of 125I-EGF—To monitor 125I-EGF internalization, cells cultured in 12-well dishes were incubated with 125I-EGF in binding medium at 37°C for 1–6 min. After indicated times, the medium was aspirated, the monolayers were rapidly washed 3 times with DMEM to remove unbound ligand and then incubated for 5 min with 0.2 nM acetic acid (pH 2.5) containing 0.2 nM NaCl at 4°C. The analog R570 was combined with another short rinse with the same acidic solution to determine the amount of surface-bound 125I-EGF. Finally, the cells were lysed in 1× NaOH to quantitate internalized radioactivity. The ratio of internalized and surface radioactivity was plotted against time and their respective rate constant for internalization kτ was calculated as linear regression. Nonlinear binding was measured for each time point in the presence of 100-fold molar excess of unlabeled EGF, and was not more than 3–7% of the total counts.

EGF Receptor Down-regulation—To monitor EGF receptor down-regulation, cells on 12-well dishes were incubated for various times with or without EGF (100 ng/ml) in binding medium at 37°C, rinsed with cold DMEM, and surface-bound EGF was removed by successive incubating the cells with ice-cold 0.2 mM sodium acetate buffer (pH 4.2) containing 0.5 mM NaCl for 20 min and 20 followed by two rinses with DMEM. This acid wash procedure did not affect binding properties of
EGF receptors. The number of binding sites on the cell surface was then determined by incubating the cells with 100 ng/ml 125I-EGF at 4 °C for 1 h.

Nonspecific binding was measured for each time point in the presence of 50-fold molar excess of unlabeled EGF, and was 5–15% of the total counts. Specific binding is reported.

Potassium Depletion of Cells—Potassium depletion of cells was performed as described by Cupers et al. In brief, cells were subjected to hypotonic shock by incubation in DMEM/water (1:1) at 37 °C for 5 min. Cells were then incubated in simplified medium (140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 5.5 mM glucose, 0.1% bovine serum albumin, 20 mM Heps, pH 7.3) devoid of KCl at 37 °C for 1 h, and subsequently with 125I-EGF (1 ng/ml) in the same medium for 1–6 min at 37 °C. Measurements of the amounts of surface and internalized radioactivity were performed as described above for internalization experiments. Control cells were incubated in simplified medium containing 10 mM KCl.

EGF Receptor Turnover—NIH3T3 expressing wild-type and mutant EGF receptors were plated in 35-mm dishes at 5 × 10^5 cells/dish. 24 h later cells were incubated at 37 °C for different times in DMEM containing 10% newborn calf serum and 100 ng/ml EGF or immediately lysed. Cell lysates were prepared as described previously. Total proteins were measured by Bio-Rad. Cell lysate (80 μg of total protein) were run on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and the EGF receptor proteins were detected by blotting with polyclonal antibody 2913 as described. Blots were exposed to Kodak X-Omat x-ray film at −80 °C and developed. Quantitation was performed using a Molecular Dynamics Computing Densitometer. Turnover rate of EGF receptors were calculated by linear regression analysis of time of incubation with EGF.

RESULTS

Mutations within the EGF Receptor Domain between Residues 972 and 977 Eliminate Receptor Interaction with AP-2—We have previously demonstrated that the carboxyl-terminal residues 973-1186 of the EGF receptor are necessary for receptor interaction with AP-2 (27). The lack of receptor-AP interaction correlated with impaired internalization and down-regulation of the receptor mutant in which these 214 residues were truncated (14, 16). The putative EGF receptor internalization sequence 973FYRAL977, located in the carboxyl terminus distal to the core tyrosine kinase domain, has similarity to a large number of endocytic signals with the more general motif of tyrosine and a hydrophobic residue (Leu) in i + 3 position (42, 43). To examine whether this motif is involved in AP-2 binding to the EGF receptor, a deletion mutant lacking residues FYR was constructed (Fig. 1). Furthermore, to analyze the role of single amino acids in this domain, Tyr974 and surrounding residues were substituted for by alanine. In addition, Tyr974 was also mutated to phenylalanine. EGF receptor single and multiple point mutants and the deletion mutant were expressed in NIH3T3 cells. The level of expression of transfected receptors in different cell clones varied from 0.8 to 9.0 receptors per cell. Three clones of NIH3T3 cells expressing 1.0 receptors in different cell clones were used as control for the mutant transfected cells.

To study interaction of EGF receptors with AP-2, the co-immunoprecipitation assay was employed. EGF receptors were immunoprecipitated, and the presence of AP-2 in immunoprecipitates was tested by blotting with antibodies specific to α-subunits of AP-2 (AC1-M11 or Ab31). In preliminary experiments, we found that the association of AP-2 with wild-type EGF receptors that had not been activated by EGF, was significantly elevated when sodium deoxycholate was included in Triton X-100-containing lysis buffer (TGH) during or after cell solubilization. Co-immunoprecipitation of AP-2 and EGF receptors recovered from EGF-treated cells was similar or even increased in the presence of sodium deoxycholate compared to co-precipitation in TGH lysates. This allowed us to measure EGF receptor/AP-2 association in cells expressing low levels of receptors. Furthermore, in the presence of sodium deoxycholate the association was EGF-independent (Fig. 2A, left panel) and could not be influenced by the different kinetics of EGF-induced endocytosis in various cell lines.

Fig. 2 shows that α-subunits of AP-2 were readily detected in immunoprecipitates of wild-type receptors. Quantitations showed that up to 5% of the total cellular pool of AP-2 was associated with EGF receptors. In contrast, virtually no AP-2 was found in immunoprecipitates of Δ973–5 mutant. Moreover, single point mutation of Tyr974 alone or together with surrounding residues eliminated co-precipitation of AP-2 with EGF receptors (Fig. 2B). Interestingly, AP-2/EGF receptor co-immunoprecipitation was highly sensitive to the conserved substitution of Tyr974 to phenylalanine (Phe974 mutant, Fig. 2A). Receptor-AP interaction was also impaired by the double mutation of Arg975 and Leu977 (R975A/L977A). These data suggested that the 973FYRAL 977 motif is critical for AP-2 binding to the EGF receptor.

Peptide 964–978 Blocks Receptor-AP Association—The ability of sodium deoxycholate to induce an in vitro AP-2/receptor association was used to test whether AP-2 binding to wild-type EGF receptors can be blocked by the soluble peptide containing FYRAL motif. TGH lysates of WT-3 cells were incubated with the synthetic peptide corresponding to the sequence His964–Tyr974, Arg975, Phe976, Met978 of the EGF receptor, and then sodium deoxycholate (1%) was added to induce receptor-AP interaction (Fig. 3). As shown in Fig. 3A, preincubation of lysates with 0.3 mM peptide 964–
978 (approximately 20,000-fold molar excess compared to the EGF receptor) blocked sodium deoxycholate-induced co-immunoprecipitation of AP-2 with EGF receptors. In parallel experiments, the peptide completely blocked co-immunoprecipitation of EGF receptors with AP-2 (Fig. 3A). Typically, a 50% inhibition of receptor-AP co-immunoprecipitation was achieved at 20–50 μM peptide (Fig. 3C). Control peptide corresponding to the residues 1168–1179 (Tyr1173) of the EGF receptor (peptide 1168–1179, 0.5 mM) did not inhibit association of AP-2 with EGF receptors (Fig. 3D) suggesting that the inhibitory effect of peptide 964–978 is specific. Notably, even very high concentrations of the specific peptide did not cause significant dissociation of neither AP-2 from the receptor immunoprecipitates (Fig. 3A) nor the receptor from AP-2 immunoprecipitates (data not shown) indicative of a very low dissociation constant of the complex. Moreover, whereas receptor-AP association in TGH lysate could be disrupted by 0.5 M NaCl, sodium deoxycholate-induced interaction was resistant to 1 M NaCl (data not shown). These results together with the data obtained with mutant receptors strongly suggest that the sequence containing Tyr1173 is the major and possibly the only high-affinity AP-2-binding site in the EGF receptor.

Importance of AP-2 Binding Site for EGF Internalization Depends on the Receptor Expression Level—To investigate the role of this AP-binding motif in EGF receptor endocytosis, the rates of 125I-EGF internalization in cells expressing wild-type and mutant receptors were compared. EGF endocytosis is a saturable process and has a maximal rate when low number of receptors are occupied by EGF (9). Therefore, 0.2 or 0.5–1.0 ng/ml 125I-EGF was used to measure internalization rates in cells expressing, respectively, high or low levels of transfected receptors. Under these conditions less than 10,000 receptors were occupied by 125I-EGF, and the contribution of the recycling of 125I-EGF receptor complexes in the uptake was insignificant (44, 45). As shown in Fig. 4A, mutation of Tyr1173 reduced the internalization rate in cells that expressed 3 x 10^5 mutant receptors. Additional mutations of Phe973, Arg975, and Asn972 to alanine caused slightly more pronounced inhibition of internalization when the receptor mutants were expressed at similarly high levels. Mutation of Pro966 and Pro968 in the context of quadruple point mutant N972A/P973A/Y974A/R975A did not cause any additional decrease of the internalization.
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Fig. 5. The dependence of internalization rates of wild-type and mutant EGF receptors from the receptor expression in the cells. The internalization rate constants ($k_i$) were calculated from $^{125}$I-EGF uptake experiments similar to those shown in Fig. 4. Each data point represents an average value from three to eight independent experiments. In all experiments no more than 10,000 receptors/cell occupied by EGF were present on the cell surface during the assay. The amount of receptors for each cell line was determined by Scatchard analysis or by measuring the specific binding using saturating concentrations of $^{125}$I-EGF (100–300 ng/ml). Dash lines indicate averaged $k_i$ values for wild-type and Dc-214. Error bars represent standard deviations. ●, WT; ▲, Δ973–5; *, Y974F; ○, Y974A; ●, F973A/Y974A; ■, F973A/Y974A/R975A; □, NA972A/F973A/Y794A/R975A; ▼, P968A/N972A/F973A/Y974A/R975A; Δ, P966A/P968A/N972A/F973A/Y974A/R975A; □, R975A/L977A; ○, Dc-214.

AP-2 binding, utilize clathrin-dependent pathway of internalization, the effect of K$^{+}$-depletion on $^{125}$I-EGF internalization was examined. K$^{+}$-depletion of the cells is known to block the assembly of coated pits and the endocytosis of receptors which use coated pits for internalization (46). As shown in Fig. 6, internalization of both WT-1 and Δ973–5 (clone 24) cells expressing low levels of receptors was inhibited in cells depleted of K$^{+}$ ions. The internalization of receptors expressed at high levels was also reduced by K$^{+}$ depletion to the level of internalization of Dc-214 receptor mutant (0.04/min) (data not shown). These experiments supported the view that Tyr$^{974}$-containing sequence is not essential for coated-pit endocytosis of EGF receptors because the receptor possesses multiple coated pit localization signals.

Effect of the Removal of AP-2 binding Site on the Down-regulation and Turnover of EGF Receptors—Previous studies on EGF receptor trafficking showed that the rate of EGF-induced internalization correlates with the rate of EGF-induced receptor down-regulation and turnover (16). In accordance with this possibility, Fig. 7A shows that down-regulation of EGF receptor mutants expressed at high levels was slower than in cells expressing similar levels of wild-type receptors. In contrast, cells expressing low levels of mutant receptors were down-regulated as efficiently as wild-type receptors (Fig. 7B). Furthermore, the turnover rate of EGF receptor mutants was also lower than that of wild-type receptors when cells expressing high level of receptors were compared (Fig. 8A). In WT-1 and WT-3 cells EGF treatment reduced the total pool of receptors by 50% within 1 and 3 h, respectively. Two to three times longer incubation with EGF was required for a 50% reduction of the cellular pool of P966A/P968A/N972A/F973A/Y974A/R975A and Δ973–5 (d7) receptors (9 and 6.5 h, respectively). In agreement with down-regulation experiments, cells expressing low levels of mutant receptors (P968A/N972A/F973A/Y974A/R975A d13 and Y974A d9) showed a turnover rate similar to wild-type receptor (1.1 and 2.0 h, respectively). These data demonstrate that elimination of the rapid internalization pathway due to the lack of AP-2 binding to the Tyr$^{974}$-containing motif may cause significant changes in the EGF receptor trafficking.

Fig. 4. $^{125}$I-EGF internalization by wild-type and mutant EGF receptors in NIH3T3 cells. Cells were incubated with 0.2–1.0 ng/ml $^{125}$I-EGF for 1–6 min, and the amount of surface-bound and internalized radioactivity was determined as described under “Experimental Procedures.” The rate of internalization is expressed as ratio of internalized and surface $^{125}$I-EGF for each time point. 3,000–10,000 surface receptors per cell were occupied by $^{125}$I-EGF during internalization assay. A, cells expressing high levels of EGF receptors. ●, WT-3; ○, WT-2; ○, Y974A; □, Y974A/F973A; ■, P966A/P968A/N972A/F973A/Y974A/R975A cl19; ■, F973A/Y974A/R975A cl19; ▲, Δ973–5 cl17. B, cells expressing low levels of EGF receptors. ●, WT-1; ○, Y974A cl19; □, N972A/F973A/Y974A/R975A cl15. The numbers of receptors per cell for individual cell clones were following: Y974A d7 = 3 × 10$^6$; Y974A d9 = 1.6 × 10$^6$; F973A/Y974A/R975A d9 = 4.0 × 10$^5$; N972A/F973A/Y974A/R975A d5 = 1.0 × 10$^5$; P966A/P968A/N972A/F973A/Y974A/R975A d9 = 5.0 × 10$^5$; Δ973–5 d7 = 4.0 × 10$^5$.

A summary of internalization experiments is presented in Fig. 5. Specific rate constant for internalization ($k_i$) was similar (0.20–0.25 min$^{-1}$) for all the three clones of cells expressing low and high levels of wild-type EGF receptor. In contrast, $k_i$ value for each receptor mutant lacking AP-binding site decreased with the increase of the mutant expression. EGF receptor mutants expressed at low levels had $k_i$ values of ~0.2/min in the range of wild-type receptor values. Minimal $k_i$ values for single and multiple point mutants and a deletion mutant expressed at high levels (0.07–0.10/min) were 2–3 times lower compared to wild-type receptors. These rates were, however, slightly higher than the internalization rate of Dc-214 receptor mutant lacking almost the entire carboxyl terminus (~0.04/min). This result implies that the carboxyl terminus contains multiple internalization signals. Therefore, data in Figs. 4 and 5 suggest that in the absence of high-affinity AP-binding motif, another mechanism supports rapid EGF internalization. However, this latter pathway is abolished in cells expressing high levels of receptors.

To test whether mutant receptors, which are incapable of
DISCUSSION

The endocytosis of EGF receptors proved to be a useful model to study mechanisms of clathrin-dependent internalization because synchronous clustering of EGF receptors in coated pits can be triggered by EGF. Indeed, EGF receptor was the first receptor shown to co-immunoprecipitate with AP-2 in cell extracts (26). The extent of co-immunoprecipitation could be affected by temperature, length of incubation with EGF, and K⁺-depletion of intact cells prior to solubilization. Therefore, it has been proposed that co-immunoprecipitation reflects an in vivo interaction of EGF receptors and AP-2 (26, 27). Recently, similar in vivo interaction of an activated Neu receptor, a tyrosine kinase of EGF receptor family, with AP-2 has been demonstrated (47). None of other receptors have been shown to co-immunoprecipitate with AP-2.

In this study, we used a modified immunoprecipitation assay to determine the AP-2 binding sequence in the EGF receptor, since the sensitivity of the assay was significantly increased by addition of the anionic detergent sodium deoxycholate to the cell lysates. The interaction of EGF receptors with AP-2 in sodium deoxycholate lysates was independent of EGF and other in vivo treatments and, therefore, was a direct measure of the intrinsic abilities of EGF receptors to bind AP-2. Presumably, sodium deoxycholate could facilitate receptor-AP association by changing the conformation of interacting components and mimicking EGF-induced conformational changes. Alternatively, sodium deoxycholate could remove a physical barrier, possibly another molecule, that prevented receptor-AP interaction in Triton X-100 extracts.

Using receptor point and deletion mutants, we found that the region containing the 973⁰YRAL 977 motif was essential for interaction of the EGF receptor with AP-2 in NIH3T3 cells (Fig. 2). Single mutation of Tyr⁹⁷⁴ was sufficient to eliminate EGF receptor/AP-2 co-immunoprecipitation. The mutations within the 966–977 domain, however, did not affect autophosphorylation and kinase activity of the receptor (data not shown), suggesting that these mutations did not cause secondary alterations in the structure of the receptor intracellular domain. During preparation of our manuscript, Nesterov et al. (48) reported that a 21-amino acid stretch within the EGF receptor (residues 970–991) is responsible for the interaction of AP-2

Fig. 6. Effect of K⁺-depletion of the cells on ¹²⁵I-EGF internalization. WT-1 and Δ973-5 cells (clone 24 cells, 1.0 × 10⁵ EGF receptors/cell) were depleted (+) or not (−) of K⁺ ions as described under "Experimental Procedures." and then the internalization rate of ¹²⁵I-EGF (1 ng/ml) was measured as described in the legend to Fig. 4 in the absence or presence of K⁺ ions. Error bars represent standard deviations from three independent experiments.

Fig. 7. Time course of down-regulation of wild-type and mutant EGF receptors. Cells were incubated with or without 100 ng/ml unlabeled EGF for indicated time, and the residual number of surface receptors was determined as described under "Experimental Procedures." Binding is expressed as percent of the ¹²⁵I-EGF binding in cells incubated without EGF. A, cells expressing high levels of EGF receptors. B, cells expressing low levels of EGF receptors. Data are representative of three independent experiments. Error bars represent standard deviations. The levels of receptor mutant expression in individual clones are indicated in the legends to Figs. 4 and 6. Symbols as in Figs. 4 and 5.

Fig. 8. Turnover of wild-type and mutant EGF receptors. Cells were incubated with 100 ng/ml EGF for the indicated time or lysed immediately. Cellular extracts (80 µg of total protein) were run on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and reacted with anti-EGFR (2913Ab). Quantitation was performed by densitometric scanning of autoradiograms. The relative number of residual receptors is expressed as percent of the number of receptors not treated with EGF (time 0). Results are the average of two independent experiments. A, cells expressing high levels of EGF receptors. B, cells expressing low levels of EGF receptors. C, R968A/N972A/F973A/Y974A/R975A d13 cells has 1.8 × 10⁵ receptors/cell. Levels of expression of other receptor mutants in individual clones are indicated in legend to Fig. 4. Symbols as in Fig. 5.
and EGF receptors. This result supports our data obtained using point mutants because FYRAL motif is located within 970–991 region.

Furthermore, to confirm the direct involvement of FYRAL motif in receptor-AP interaction, we demonstrated that the soluble 15-amino acid peptide containing this motif blocked AP-2 association with the wild-type EGF receptor (Fig. 3). The relatively low inhibition constant (20–50 μM) and the inability of peptide 964–978 to dissociate the preformed receptor-AP complex (Fig. 3) is indicative of the specificity and high affinity of AP-2 interaction with Tyr974-containing motif. Therefore, our data are in agreement with the studies of an in vitro interaction of bovine brain AP-2 with constitutively-internalized proteins which revealed the importance of endocytic sequences for AP-2 binding (22–25). In our experiments, however, EGF receptor-AP-2 complexes were formed in whole cell lysates at concentrations of receptors and AP-2 several magnitudes lower than those used in the above mentioned studies. Moreover, receptor-AP complex was resistant to a high salt treatment, a condition that dissociates EGF receptor complex with proteins containing src-homology 2 domains (data not shown).

The EGF receptor-AP interaction was sensitive to the conserved Tyr → Phe mutation (Phe974 mutant), suggesting the importance of the hydroxyl group of Tyr974 for the three-dimensional structure of AP-binding domain. Perhaps, the conformation of the EGF receptor internalization domain in the AP-bound state is different from that assumed in solution, and the hydroxyl group may be critical to support the AP-bound conformation. A similar Tyr → Phe mutation within the internalization domain of lysosomal acid phosphatase inhibited lysosomal acid phosphatase endocytosis (49). NMR studies of this lysosomal acid phosphatase domain predicted that the Tyr → Phe mutation decreased the tendency to form a β-turn conformation of the domain by 25% (49). Thus, AP-2 interaction with EGF receptors may be a valid model to study the structural aspects of high-affinity receptor-AP interactions.

It is striking that other regions of the large carboxyl-terminal tail of the EGF receptor which have internalization consensus signals and a predicted tight turn conformation (17) did not bind AP-2 in our experiments. Probably, proper position within the cytoplasmic tail and surrounding amino acids are essential for the high-affinity interaction of internalization sequences with AP-2. The possibility cannot be ruled out that other experimental conditions are required to reveal AP-2 binding to other regions. The sensitivity of our assay in the presence of sodium deoxycholate would not allow to detect AP-2 co-internalization with the mutant receptors that is less than 5–10% of the amount of AP-2 co-precipitated with the wild-type receptor. However, AP-2 did not bind to EGF receptor mutant lacking 959–992 residues nor fusion proteins corresponding to other parts of the receptor in the absence of sodium deoxycholate (48).

The FYRAL sequence have been shown to be sufficient for the rapid saturable endocytosis of an EGF receptor mutant that lacks the entire carboxyl-terminal domain (17, 18). The latter studies, however, did not reveal whether this domain functions in the full-length EGF receptor. To address this question, we analyzed the effect of single point mutations or small internal deletion within this motif on the internalization of the full-length receptor. Unexpectedly, the importance of this motif was dependent on the total amount of receptors expressed in the cell, although all experiments were performed under conditions when similarly low numbers of surface receptors were occupied by 125I-EGF (Figs. 4 and 5). The rate of internalization was reduced 2–3 times compared to wild-type receptors in cells expressing high level of the receptor mutants (ki = 0.07–0.13/min versus 0.20–0.25/min), whereas it was not affected in cells with low levels of the same mutants (ki = 0.20–0.22/min). The minimal rates of 125I-EGF internalization by the receptor mutants were comparable to the internalization rate of unoccupied wild-type receptors in NIH3T3 cells (ki = 0.08–0.10/min). Therefore, AP-2 binding to FYRAL motif is important for the EGF-induced pathway of internalization in cells expressing high levels of receptors. As expected, loss of this pathway resulted in impaired receptor down-regulation and turnover (Figs. 7 and 8). Our data agree with the observation that the removal of the FYRAL domain, as part of a large deletion of residues 959-1022 or 959–992, did not affect 125I-EGF internalization (17, 48). However, the latter result was obtained in cells expressing low number of receptors.

Therefore, it can be proposed that the high-affinity binding of EGF receptors to AP-2 is one of the mechanisms by which EGF-occupied receptors are recruited into coated pits. The mechanism(s) that support rapid internalization of mutant receptors, incapable of high-affinity AP-2 binding, remain to be determined. Data in Fig. 6 suggested that these mutant receptors used coated pits for internalization. It is possible that EGF-activated mutant receptors were recruited into coated pits through low-affinity interactions with AP-2 that are induced by EGF and mediated by sequences other than FYRAL. However, unoccupied receptors are also capable of weak association with AP-2 (26, 28) and can compete with EGF-activated receptors for AP-2 binding. Large excess of unoccupied receptors might inhibit binding of a small pool of 125I-EGF-occupied receptors to AP-2. This competition would result in slow internalization of 125I-EGF in cells expressing high levels of receptor mutants as observed in our experiments (Figs. 4 and 5). In contrast, EGF-induced high-affinity interaction of wild-type EGF receptors with AP-2 via FYRAL motif would dominate in the competition with unoccupied receptors regardless of the level of receptor expression in the cell.

Alternatively, rapid EGF-induced endocytosis of mutant receptors can be mediated by protein(s) other than AP-2. These hypothetical “coated pit recruiter(s)” might be present in limited amount in the cell and constitutively associated with a limited pool of EGF receptors in the absence of EGF. The increase in the expression level of receptors would titrate the putative recruiter, and this would increase the relative importance of the pathway that requires direct receptor-AP interaction. In cells expressing low levels of receptor mutants, the extent of association of EGF-occupied receptors with the recruiter molecules would be relatively high resulting in a high rate of endocytosis, independent of the AP-2 pathway. The contribution of independent pathway could depend on the expression of this putative recruiter and may be very different cell lines.

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