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Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins

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ABSTRACT **Ligand-activated epidermal growth factor receptors (EGFRs) associate with coated pit adaptor proteins (AP2) *in vivo*, implying a mechanism for receptor retention in coated pits during internalization. Using an *in vitro* binding assay, we localized the adaptor binding determinant to residues 970–991 of EGFRs and confirmed specificity by competition with a synthetic peptide corresponding to this sequence. A mutant EGFR lacking this AP2 binding determinant did not associate with AP2 *in vivo* but demonstrated internalization and down-regulation kinetics indistinguishable from its wild-type counterpart. Immunocytochemistry confirmed ligand-induced internalization of the mutant EGFR. These data suggest that endocytic determinants are distinct from AP2 binding determinants and that processes other than association with AP2 regulate endocytosis of EGFRs.**

Adaptor proteins anchor clathrin lattices to the cytosolic face of the plasma membrane and are an important structural component of coated pits (1, 2). The plasma membrane adaptor (AP2) is a heterotetramer consisting of two ≈100-kDa proteins (α- and β-adaptins) and two smaller subunits of 50 and 16 kDa (3). The subunit composition distinguishes AP2 from the Golgi complex adaptor AP1. It has been proposed that, in addition to a well-established structural function involved in clathrin assembly and coated pit formation (1–5), AP2 complexes also participate in specific retention of receptors in coated pits and thus regulate their endocytosis (1, 6). In support of this hypothesis, *in vitro* binding studies have shown interaction of AP2 with the cytoplasmic domains of the low density lipoprotein receptor, the mannose 6-phosphate receptor, the asialoglycoprotein receptor, and lysosomal acid phosphatase (6–9). Binding *in vitro* appeared to require defined cytoplasmic sequences initially identified by their requirement for receptor internalization *in vivo*. These endocytic determinants or "codes" consist of 4–6 amino acids including an essential aromatic residue that adopt a tight turn configuration (10–14). Despite requisite specificity, the affinity of AP2 binding to receptor sequences *in vitro* is low and of unproven functional importance. A significant observation made by Sorkin and Carpenter (15) was that the epidermal growth factor receptor (EGFR) binds AP2 *in vivo* with high affinity in a temperature- and EGF-dependent fashion. It was subsequently shown that binding occurs through direct interaction between AP2 and EGFR molecules (16) and is regulated by tyrosine autophosphorylation of EGFR that introduces a conformational change in the receptor molecule, which exposes AP2 binding determinants (17).

Two lines of evidence indicate that the AP2 binding region is found in the regulatory C terminus of EGFRs. Deletion of the C terminus abolishes AP2 binding *in vivo*, whereas high-affinity association between a recombinant EGFR C terminus and purified AP2 can be shown *in vitro* (16, 17). Because the three regions responsible for EGFR endocytosis are also found in the same 228-amino acid regulatory C terminus located distal to the tyrosine kinase core (18), precise mapping of an AP2 binding motif was required to evaluate its role in endocytosis.

In the present studies, we identified a 22-amino acid AP2 binding determinant in the C terminus of the EGFR molecule and found that deletion of this motif abolishes detectable formation of EGFR–AP2 complexes *in vivo*. Surprisingly, an EGFR mutant deficient in AP2 binding is internalized and down-regulated with the same kinetics as its wild-type (WT) counterpart. These results suggest that processes other than adaptor-mediated retention in coated pits are required for EGFR endocytosis.

MATERIALS AND METHODS

Materials. Mouse monoclonal antibody 528, specific to the extracellular domain of human EGFR (19), was from H. Masui (Memorial–Sloan Kettering, New York); rabbit polyclonal antibody 4516-8, specific to residues 647–688 of human EGFR (20), was from T. Hunter (The Salk Institute, La Jolla, CA); monoclonal antibody AC1-M11, specific to α-adaptin (21), was from M. Robinson (Cambridge University, Cambridge, U.K.); mouse monoclonal anti-phosphotyrosine antibody PY-20 was from Transduction Laboratories (Lexington, KY). Adaptor complexes isolated from bovine brain (22) were generously provided by S. Schmid (Scripps Institute, La Jolla, CA).

Construction and Expression of Mutant EGFR. To generate the fusion plasmid c'958f993–1186 EGFR, *Sal* I sites were created at residues 959 and 992 by site-directed mutagenesis. The sequences between the two *Sal* I sites were deleted, and valine and aspartate residues were introduced at the junction. The construction was verified by dideoxynucleotide sequencing. Residue 958, corresponding to an exon–intron junction, represents the C terminus of the functional tyrosine kinase domain; residue 1186 is the C terminus of WT EGFR. Clonal B82 cells expressing both WT and mutant EGFR constructs were prepared and maintained as described (18, 23).

Construction and Expression of Glutathione *S*-Transferase (GST) Fusion Proteins. Fragments of DNA encoding various regions of EGFRs were amplified by using *Pfu* polymerase and oligonucleotides that contained *Eco*RI and *Bam*HI restriction sites to allow oriented subcloning. The exact sequences of oligonucleotides are available from the authors upon request. The amplified DNA was subcloned in-frame into a pGST vector, which is analogous to the commercially available vector pGEX-2T (Pharmacia) except that the polypeptide EILEDERAS was added to the N terminus of the fusion protein. All constructions were verified by dideoxynucleotide sequencing. GST fusion proteins were expressed by standard techniques; bacterial lysates were pre-

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; WT, wild type; GST, glutathione *S*-transferase; TfR, transferrin receptor.

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pared by using 0.2% lauroylsarcosine, supplemented with 1% Nonidet P-40, and stored at −70°C.

Coimmunoprecipitation and Affinity Precipitation. Cells in 100-mm dishes were incubated for 1 h in ice-cold Dulbecco's modified Eagle's medium containing 20 mM Hepes (pH 7.4) without or with 200 ng of EGF per ml. Potassium depletion of cells followed the procedure described by Sorkin and Carpenter (15). Cells were placed at 37°C for 12 min and lysed with IP buffer [50 mM Hepes, pH 7.4/1% Triton X-100/10% (vol/vol) glycerol/100 mM NaCl/10 mM NaF/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1 mM Na_3VO_4/10 mM benzamidine/10 μg of aprotinin per ml/10 μg of leupeptin per ml] and EGFR was immunoprecipitated with monoclonal antibody 528.

For affinity precipitation of AP complexes, 10 μl of glutathione-agarose beads was incubated with 200 μl of bacterial lysates containing the indicated GST–EGFR fragments for 1 h at 4°C. The concentration of lysate was adjusted to allow capturing of approximately equal amounts of GST fusion protein (30–50 μg). Beads were then washed twice with IP buffer containing 25 mg of bovine serum albumin per ml (IP/BSA). AP complexes (30 μg/ml) were spun for 30 min at 100,000 × *g* to remove aggregates and incubated with beads in 300 μl of IP/BSA for 1 h at 4°C with continuous agitation. Beads were then washed once with IP/BSA and three times with IP buffer.

In some experiments, competitive elution of bound AP was carried out with a synthetic peptide. Immunobeads, containing immunoprecipitated EGFR or GST beads with associated AP complexes, were washed as described above, and 1 mM synthetic peptide corresponding to EGFR residues 970–991 (*KK*DSNFYRALMDEEDMDDVVDADE) in 300 μl of IP/BSA was added. After 1 h of continuous agitation at 4°C, beads were washed once with IP/BSA and once with IP buffer.

Material bound to beads was resolved by electrophoresis and probed with rabbit polyclonal antibody 4516-8 or mouse monoclonal antibody AC1-M11 or PY-20. To sequentially probe the same filter with different antibodies, it was stripped as described (17). Detection was via enhanced chemiluminescence with peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit immunoglobulin.

Internalization and Down-Regulation of EGFRs. B82 cells expressing WT or mutant EGFR were switched to serum-free medium 18 h before experiments. Binding experiments were initiated by changing to medium containing ^{125}I-labeled EGF (^{125}I-EGF) (24). At each time point, the relative amounts of ligand associated with the cell surface and interior were determined by acid stripping at 4°C using 50 mM glycine·HCl/100 mM NaCl/2 mg of polyvinylpyrrolidone per ml/2 M urea, pH 3.0. Nonspecific binding was determined with B82 cells that lack EGFR and was <1% of total binding. Specific internalization rates were determined by plotting the integral of surface-associated ligand against the amount internalized. Saturation of specific receptor internalization was established with ^{125}I-EGF concentrations ranging from 1 to 120 ng/ml. The internalization velocity was plotted against the specific internalization rate, yielding a Satin plot (25).

Down-regulation was determined by treating cells with 50 nM EGF for the indicated times at 37°C. Dishes were placed on ice and surface-bound EGF was stripped by incubation in 50 mM acetic acid (pH 2.5) containing 135 mM NaCl and 2.5 mM KCl for 5 min. After rinsing, residual surface receptors were detected by incubation with 1.0 nM ^{125}I-EGF at 4°C for 4 h (24). Nonspecific binding was determined in the presence of 200-fold molar excess of unlabeled EGF and was <5%. Data represent means ± SE from three experiments each containing triplicate dishes at each data point.

Immunofluorescence Microscopy. Cells plated on fibronectin-coated coverslips 48 h previously were treated without or with 100 ng of EGF per ml for 2 h and were then fixed for 15 min with freshly prepared 3% paraformaldehyde/0.02% glutaraldehyde and then permeabilized for 15 min with 0.0125% saponin. Free aldehyde groups were quenched with 0.1% $NaBH_4$ for 10 min. Cells were incubated with a mixture of anti-EGFR monoclonal antibodies 528, 579, and 225 (10 μg/ml each) for 1 h followed by staining with fluorescein isothiocyanate-labeled goat anti-mouse antibodies (1:100) for 45 min. The coverslips were mounted in ProLong antifade medium (Molecular Probes) and viewed with a Nikon inverted fluorescence microscope with a ×100 oil immersion objective. Images (512 × 512) were acquired using a Photometrics (Tucson, AZ) cooled CCD camera and a Macintosh workstation running ONCORIMAGE software. Images were scaled to 256 grey levels using Adobe PHOTOSHOP 3.0 on the Macintosh before output to a film recorder.

RESULTS

***In Vitro* Mapping of the Site of Interaction of EGFR with AP2.** Removal of the C terminus of EGFR distal to the protein tyrosine kinase domain abolished both ligand-induced receptor endocytosis (23) and *in vivo* association of EGFR with AP2 detected by coimmunoprecipitation (16, 17). Because the C terminus of EGFR contains three regions that can independently function in endocytosis (18), these were candidate regions to contain adaptor binding determinants. To identify the region responsible for association of EGFR and AP2, a series of *in vitro* binding reactions were carried out using either the holocarboxyl terminus of EGFR or separate endocytosis-defining regions expressed as GST fusion proteins (Fig. 1*A*). Equal amounts of each GST fusion protein were assessed for their ability to bind purified AP2 complexes (Fig. 1*B*). All the constructs containing the endocytosis determinant in region I, which encompasses EGFR residues 970–991, bound AP2 with high affinity. Surprisingly, EGFR regions II and III, which function in endocytosis with better efficiency than region I (18), were unable to bind AP2. The integrity of region I appeared to be important for AP2 binding because deletion of five residues from its N terminus (GSTf975–991) or eight residues from its C terminus (GSTf970–983) drastically reduced AP2 binding. Because EGFR residues 973–977 of region I functioned efficiently in endocytosis when substituted for the endogenous endocytic code of the transferrin receptor (TfR) (18), these results suggest that the EGFR adaptor binding determinant is overlapping but distinct from the endocytic codes of EGFR.

An Identified 22-Amino Acid Fragment of EGFR Required for Interaction with AP2 *in Vivo*. The 22-amino acid fragment of EGFR that bound AP2 *in vitro* is predicted to form a turn–helix structure in holo-EGFR (23). To confirm that the adaptor binding region is found between residues 970 and 991 of EGFR, we attempted to specifically displace bound AP2 with a synthetic peptide corresponding to this region. As shown in Fig. 2 (affinity precipitation) purified AP2 was specifically bound to the immobilized GST fusion protein containing the entire EGFR C terminus (amino acids 970–1186). Incubation of the complex with the synthetic peptide representing EGFR residues 970–991 eluted the majority of bound AP2. Inclusion of various concentrations of peptide during the incubation gave a half-maximal displacement at 120 μM. In control experiments, a synthetic peptide representing residues 1164–1176 of EGFR gave no significant displacement of AP2 (data not shown).

To determine whether the peptide could effectively disrupt the complex formed between activated EGFR and AP2 *in vivo*, A431 cells were treated without or with EGF, EGFRs were immunoprecipitated with antibody 528, and immunoprecipitates were incubated without or with the synthetic peptide. As shown in Fig. 2 (coimmunoprecipitation) incubation with the synthetic peptide was sufficient to elute the majority of bound

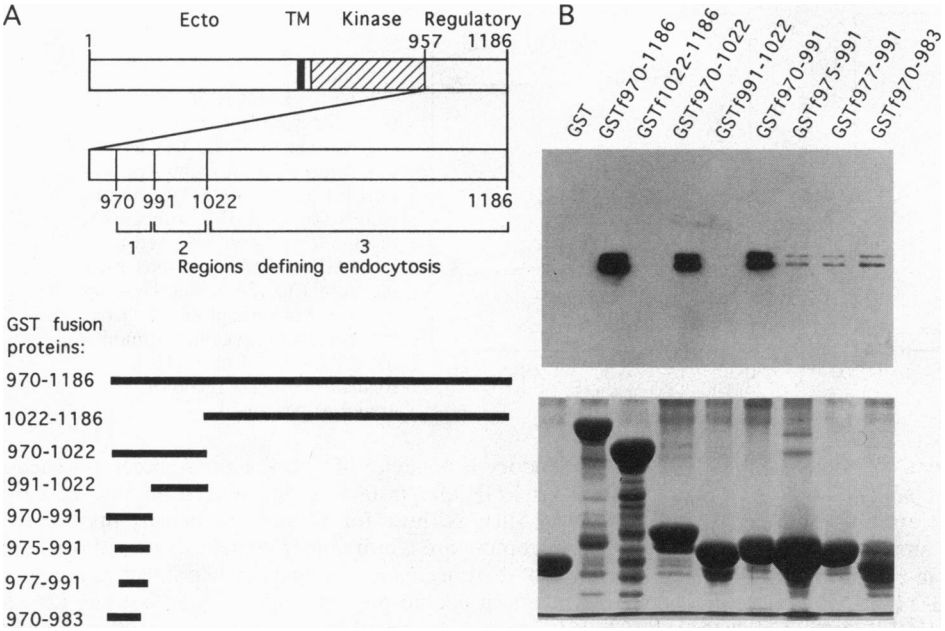

FIG. 1. Binding of AP2 to EGFR fragments. (*A*) Schematic representation of EGFR C-terminal regions that determine endocytosis and corresponding GST fusion proteins used in binding studies. (*B*) GST fusion proteins representing various regions of the C terminus of EGFRs were incubated with isolated AP complexes. One-half of bound material was then either analyzed by Western blotting with antibody to α-adaptin (*Upper*) or resolved by electrophoresis and stained for total protein to quantitate GST fusion proteins (*Lower*).

AP2. The control peptide corresponding to residues 1164–1173 of region III of the EGFR C terminus was without effect (data not shown).

Both *in vitro* mapping and competition with peptide identified a 22-amino acid region of EGFR as the site of interaction with AP2. To more critically examine the identified site of high-affinity interaction of EGFR with AP2, an EGFR mutant lacking this region was constructed. Residues 959–992, which include the adaptor binding motif, were deleted and a mutant EGFR designated as c′958f993–1186 was expressed in B82 cells that lack endogenous EGFR. Mutant EGFRs were expressed at the same level as their WT counterpart (2.5×10^5 receptors per cell). Because this mutant lacks only one of the five identified autophosphorylation sites (residue 992) it exhibited approximately the same level of tyrosine kinase activity, as judged by ligand-induced autophosphorylation (Fig. 3). Cells expressing WT and mutant EGFRs were treated without or with EGF followed by EGFR immunoprecipitation. As shown in Fig. 3, deletion of residues 959–992 abolished the ability of ligand-activated EGFRs to bind AP2 *in vivo*. Because the apparent failure of the mutant receptor to associate with adaptors could, in principle, be due to altered kinetics of endocytosis, we also used K^+-depletion conditions, which inhibit internalization without preventing EGFR association with AP2 (15). In contrast to ligand-stimulated WT EGFR, no association of adaptors with the mutant EGFR was detected under conditions of K^+ depletion. Binding of AP2 to EGFR thus requires ligand activation and a specific sequence determinant in the C terminus of EGFR.

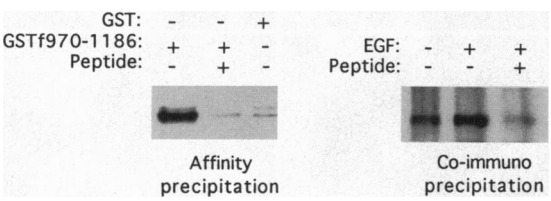

FIG. 2. Competitive displacement of bound AP2. For affinity precipitation, glutathione-agarose beads containing either GST fused to residues 970–1186 of EGFR or GST alone were incubated with isolated AP complexes. For coimmunoprecipitation, EGFR–AP2 complexes were immunoprecipitated with antibody 528 from A431 cells treated without or with EGF (200 ng/ml). After extensive washing, both glutathione beads and immunobeads were incubated for 1 h with 1 mM synthetic peptide representing residues 970–991 of EGFR or with buffer alone. AP2 complexes bound to the beads were detected by Western blotting with antibody to α-adaptin. Data are representative of five independent experiments.

Comparison of Endocytosis and Down-Regulation of WT and Mutant EGFRs Defective in Adaptor Binding. The kinetics of endocytosis and down-regulation of the mutant EGFRs defective in adaptor binding were compared to those of ligand-activated WT EGFRs. Internalization rates were measured at various EGF concentrations and are presented as saturation-internalization (Satin) plots (25). In this approach, the rate of endocytosis, reflecting the probability of a single receptor molecule being internalized, is expressed as a function of the net internalization velocity, a measure of the bulk amount of receptor molecules participating in endocytosis. If any component of the endocytic system is limiting, saturation occurs, which is reflected in a negative slope of the Satin plot.

As shown in Fig. 4*A*, the endocytic rate constant (K_e) of WT EGFR decreased with increasing velocity, whereas a mutant

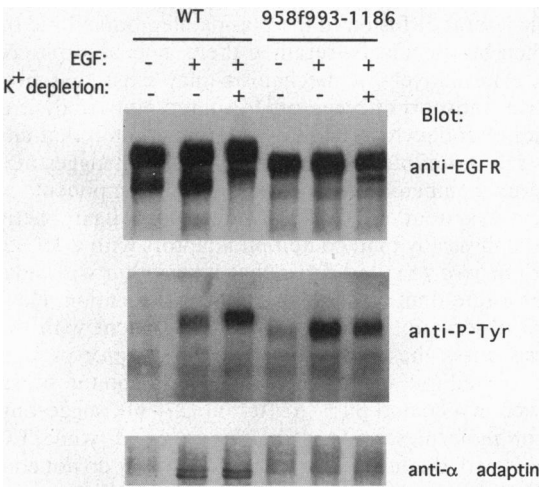

FIG. 3. Coimmunoprecipitation of AP2 with EGFR. B82 cells expressing WT or c′958f993–1186 EGFRs were treated without or with EGF (200 ng/ml) for 1 h at 4°C and then placed at 37°C for 12 min. Where indicated, cells were subjected to K^+ depletion. EGFRs were immunoprecipitated and immunoprecipitated material was then analyzed by electrophoresis and Western blotting. The same filter was consecutively probed with antibodies to α-adaptin, phosphotyrosine, and EGFR.

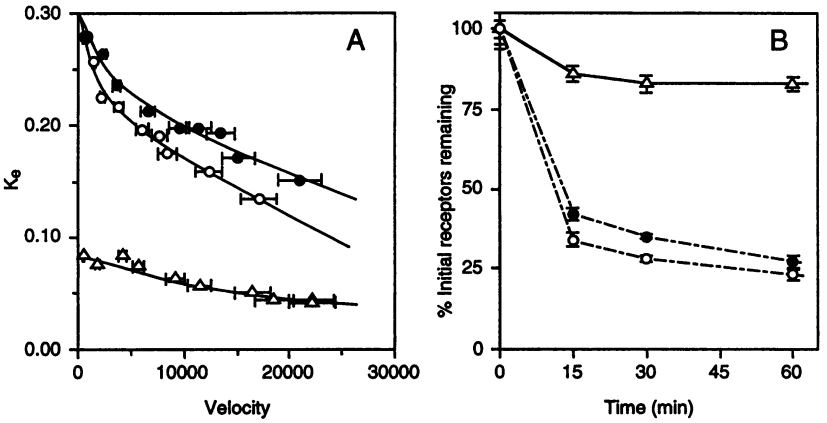

FIG. 4. Kinetics of internalization and down-regulation of WT and mutant EGFRs. (*A*) Saturation-internalization plots for WT EGFR (○), c'958f993–1186 mutant EGFR (●), or EGFR truncated to residue 958 (△) determined using ^{125}I-EGF at concentrations ranging from 1 to 120 ng/ml. Data are presented as Satin plots (25). Error bars represent range in the number of cells per plate. (*B*) Down-regulation of these receptors determined as described.

EGFR (c'958) that lacks the regulatory C terminus exhibited a low K_e independent of velocity. The c'958f993–1186 EGFR was indistinguishable from WT EGFR exhibiting a K_e of 0.3 min^{-1} at low EGF concentrations and an approach to saturation at increasing velocities. As shown in Fig. 4*B*, the rate and extent of down-regulation of c'958f993–1186 EGFR were the same as that of WT EGFR. c'958 EGFR exhibited little down-regulation, confirming previous reports (18, 23).

Ligand-dependent trafficking of the mutant EGFR was also assessed using immunocytochemistry. As shown in Fig. 5 *Left*, both WT and c'958f993–1186 EGFRs were distributed at the cell surface, consistent with low rates of endocytosis relative to rates of recycling of unoccupied receptors (26). After addition of EGF, both receptors were cleared from the surface and appeared in perinuclear endosomes and lysosomes (Fig. 5 *Right*). Endocytosis and cellular distribution of receptors without or with the high-affinity AP2 binding site were morphologically indistinguishable. These results suggest that high-affinity binding to adaptors is not required for ligand-induced receptor internalization and retention in intracellular compartments.

DISCUSSION

Two plausible mechanisms for receptor-mediated endocytosis have been considered. Receptors may reach coated pits due to chaotic lateral diffusion in the plasma membrane lipid bilayer and then be specifically retained there by coated pit components. Alternatively, a mechanism may exist that provides selective transport of receptors to coated pits. Analysis of the kinetics of endocytosis of EGFRs demonstrated that endocytosis is a saturable, second-order process, suggesting that receptors compete for an unidentified component of the endocytic system (25). The finding that ligand-activated EGFRs physically bind coated pit adaptors with a 1:1 stoichiometry *in vivo* (16) indicated that association with adaptors may be a rate-limiting step for coated pit retention. However, several observations are not readily consistent with this hypothesis. First, the estimated amount of adaptor protein in a single coated pit exceeds the maximal amount of EGFR observed in a coated pit 5- to 10-fold (27–30), suggesting that adaptor molecules are not limiting. Second, while EGFRs compete with themselves for endocytosis, they do not compete with TfR despite the fact that both receptors are internalized via the same coated pit (24, 31–33). This result indicates specificity in the endocytic machinery. Consequently, it appears unlikely that either a single molecule, such as AP2, can bind a variety of receptors by different mechanisms or that a single coated pit can accommodate a large enough pool of different molecules to provide specific retention of a variety of receptors.

The endocytic behavior of tyrosine kinase receptors such as those for EGF and insulin is different from that of cargo receptors such as those for Tf and low density lipoproteins. Cargo receptors are continuously present in coated pits independent of their occupancy by ligand, while signaling receptors concentrate in coated pits only upon ligand binding (24, 28, 34–36). Internalization of both types of receptors requires endocytic codes (14). It is proposed that in the case of cargo receptors the cytoplasmic endocytic code sequences are continuously exposed, while in signaling receptors activation of intrinsic tyrosine kinase and subsequent autophosphorylation and coformational changes are required for exposure of previously cryptic sequences (37, 38). Importantly, in contrast to signaling receptors, internalization of cargo receptors such as TfR does not appear to saturate the endocytic apparatus (25, 39). However, internalization of both types of receptors may occur through similar mechanisms because their endocytic codes are functionally interchangeable (18). Moreover, EGFR mutants in which the tyrosine kinase domain has been deleted and the endocytosis-defining regions fused directly to the juxtamembrane region are internalized in an occupancy-independent and nonsaturable fashion, similar to cargo receptors (38). Thus, the rate-limiting step observed in occupancy-induced endocytosis of tyrosine kinase receptors probably

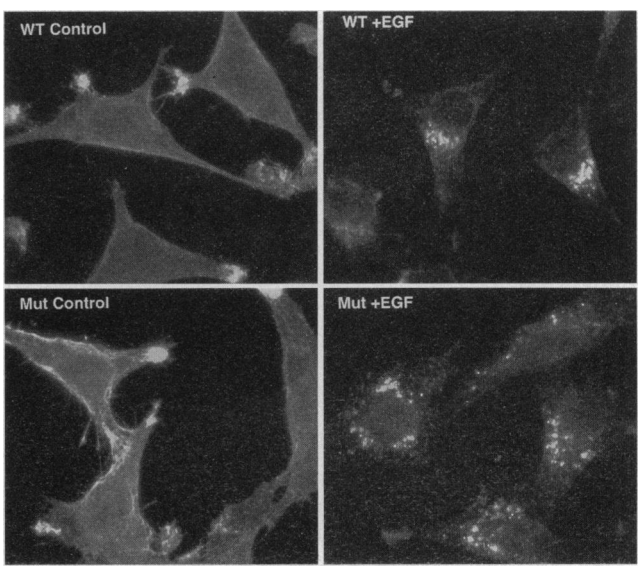

FIG. 5. Immunocytochemical analysis of WT and c'958f993–1186 EGFRs. B82 cells expressing WT or mutant EGFRs treated without or with EGF (100 ng/ml) were fixed and EGFR was detected by using antibodies specific to the ectodomain of EGFR. Detection was via fluorescein isothiocyanate-labeled goat anti-mouse antibodies. (*Left*) Without EGF. (*Right*) With EGF.

reflects competition not for coated pit components but instead for an event upstream of this process.

The present data argue against an adaptor binding function for the endocytic codes of EGFRs. The adaptor binding domain of EGFRs lies between residues 970 and 991 and is separate from the strong endocytosis-defining regions 991–1022 and 1023–1186. The adaptor binding region includes an endocytic code $N^{972}FYRAL^{977}$ defined by its ability to substitute functionally for the endogenous code of TfR (18), but integrity of the whole 22-amino acid region is required for binding to AP2. While this endocytic code constitutes part of the adaptor binding motif, it appears to function independently of this binding and alone is insufficient to bind AP2 with high affinity. In agreement with the concept that endocytic codes function independently of high-affinity adaptor protein binding, deletion of the adaptor binding motif of EGFRs abrogated association of adaptors with EGFRs *in vivo* but did not alter the ligand-induced endocytic behavior of EGFRs.

The sequence of the identified AP2 binding region of EGFRs corresponds to the most highly conserved segment of the C terminal domain of the structurally related erbB2 and erbB4 receptors (40, 41). Search of the data bases using the BLAST programs (42) revealed that high homology was restricted to these related receptors and to EGFRs from chicken, mouse, and human. Interestingly, the mutation in the transmembrane domain of erbB2 that results in constitutive activation of this tyrosine kinase receptor also results in *in vivo* association with AP2 (43). The identified AP2 binding motif of EGFRs is not conserved in kinase-inactive erbB3, nor is it conserved in insulin receptor (InsR) and TfR. We have been unable to detect adaptor association with ligand-activated InsR, with TfR, or with chimeric TfR containing EGFR endocytic codes I and II in receptor immunoprecipitates (data not shown). These receptors displayed normal endocytic behavior, suggesting that high-affinity interaction with AP2 may be restricted to a subset of receptors.

In vivo association with AP2 required ligand activation of EGFRs and intrinsic protein tyrosine kinase activity (17). Despite mechanisms similar to those proposed for exposure and activity of cryptic endocytic and lysosomal targeting codes (38, 44), both kinetic and morphologic criteria indicate that the AP2 binding region of EGFRs is not required for receptor trafficking. The data suggest that retention of EGFRs in coated pits via interaction with AP2 does not explain occupancy-induced receptor internalization and subsequent intracellular trafficking. Endocytic codes may interact weakly with AP2 and multiple weaker interactions may help to concentrate receptors in coated pits. The high-affinity interaction of EGFRs with AP2 may serve a secondary tethering function or may parallel other protein–protein interactions involving this domain.

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