All ErbB Receptors Other Than the Epidermal Growth Factor Receptor Are Endocytosis Impaired*

(Received for publication, September 15, 1995, and in revised form, November 30, 1995)

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Four transmembrane tyrosine kinases constitute the ErbB receptor family: the epidermal growth factor (EGF) receptor, ErbB-2, ErbB-3, and ErbB-4. We have measured the endocytic capacities of all four members of the EGF receptor family, including ErbB-3 and ErbB-4, which have not been described previously. EGF-responsive chimeric receptors containing the EGF receptor extracellular domain and different ErbB cytoplasmic domains (EGF/ErBb) have been employed. The capability of these growth factor–receptor complexes to mediate 125I-EGF internalization, receptor down-regulation, receptor degradation, and receptor co-immunoprecipitation with AP-2 was assayed. In contrast to the EGF receptor, all EGFR/ErbB receptors show impaired ligand–induced rapid internalization, down-regulation, degradation, and AP-2 association. Also, we have analyzed the heregulin–responsive wild-type ErbB-4 receptor, which does not mediate the rapid internalization of 125I-heregulin, demonstrates no heregulin–regulated receptor degradation, and fails to form association complexes with AP-2. Despite the substantial differences in ligand–induced receptor trafficking between the EGF and ErbB-4 receptors, EGF and heregulin have equivalent capacities to stimulate DNA synthesis in quiescent cells. These results show that the ligand–dependent down-regulation mechanism of the EGF receptor, surprisingly, is not a property of any other known ErbB receptor family member. Since endocytosis is thought to be an attenuation mechanism for growth factor–receptor complexes, these data imply that substantial differences in attenuation mechanisms exist within one family of structurally related receptors.

The epidermal growth factor (EGF) receptor is a transmembrane glycoprotein possessing a cytoplasmic tyrosine kinase active site, which is activated by specific growth factor binding to an external ligand–binding domain (1, 2). Three additional transmembrane molecules have considerable sequence homology to the EGF receptor. These four transmembrane tyrosine kinases constitute the ErbB receptor family: ErbB-1 or the EGF receptor, ErbB-2 (3), ErbB-3 (4), and ErbB-4 (5).

Ligands that specifically bind to and activate the EGF receptor include EGF and several EGF-like active products (6). Activation of the EGF receptor by ligand binding includes receptor dimerization, activation of intrinsic receptor tyrosine kinase activity, autophosphorylation of the receptor carboxyl terminus, and tyrosine phosphorylation of and/or association with intracellular signaling molecules (1, 2, 7). Ligands that specifically bind to the EGF receptor do not directly interact with ErbB-2, ErbB-3, or ErbB-4. While a specific ligand for ErbB-2, a putative receptor, has not been identified, the heregulin family of growth factors, which do not interact with the EGF receptor or ErbB-2, bind with low affinity to ErbB-3 (8, 9) and with high affinity to ErbB-4 (9). Although heregulins do not directly interact with ErbB-2 by itself, heterodimers of ErbB-2 and ErbB-3 are reported to constitute a second high affinity binding site for heregulin (10).

Binding of EGF to the EGF receptor at 37 °C rapidly induces the clustering of ligand–receptor complexes in coated pits, internalization of the complexes, and ultimately lysosomal degradation of both EGF and its receptor (11). The endocytic pathway, therefore, may function as a mechanism for the gradual attenuation of plasma membrane signaling complexes (12). While a molecular mechanism for the rapid internalization of growth factor–receptor complexes has not been established, recent evidence suggests that direct interaction of a plasma membrane dithrin-associated protein complex, termed AP-2 for adaptor protein, with the carboxyl terminus of the EGF receptor may facilitate receptor internalization through coated pits (13). AP-2 (14) is a heterotetramer of two large subunits, α and β (100–115 kDa), a medium subunit, μ2 (50 kDa), and a small subunit, ε2 (17 kDa). The nature of AP-2 interaction with the EGF receptor carboxyl terminus is not clear, but the interaction is stoichiometric, direct, and requires receptor kinase activity (15, 16). However, SH2 domains and phosphotyrosine residues seem not to be direct mediators of this association, as AP-2 subunits do not contain SH2 or SH3 domains.

Using a chimeric receptor composed of the extracellular EGF receptor binding domain and the cytoplasmic domain of the ErbB-2 molecule, one study (17) showed that EGFR/ErbB-2 receptors internalized 125I-EGF severalfold more slowly than the EGF receptor. This study also indicated that the impaired internalization capacity of this receptor was due to sequences in the ErbB-2 carboxyl–terminal domain. A more recent study (18) showed that the wild-type ErbB-2 receptor failed to associate with AP-2 and was not internalized. Surprisingly, however, the oncogenic form of ErbB-2, neu, did form a constitutive interaction...
complex with AP-2 and was internalized. In this study, we have measured the internalization and AP-2 association capacities of all four members of the EGF receptor family, including ErbB-3 and ErbB-4, which have not been described previously. We have used both chimeric receptors, containing an EGF receptor extracellular domain with each of the ErbB cytoplasmic domains, as well as the wild-type ErbB-4 receptor that responds to heregulin.

**EXPERIMENTAL PROCEDURES**

Materials—EGF was isolated from mouse submaxillary glands according to the method of Savage and Cohen (19) and iodinated as described by Carpenter and Cohen (20). Heregulin β3 (21) was obtained by thrombin cleavage of a heregulin β3 GST fusion protein expressed in bacteria. The HRG-β3 EGF domain (nucleotide position 1023–1218 in Ref. 21) was amplified by PCR to contain a 5′ BamHI and 3′ EcoRI site and, following restriction, was directionally cloned in-frame with the glutathione S-transferase coding sequence of pGEX-KT (Pharmacia Biotech Inc.). Authenticity of the open reading frame was verified by sequence analysis on both strands of the PCR-amplified fragment in the expression vector. The EGF domain of HRG-β3 was released by thrombin cleavage of the fusion protein immobilized on glutathione beads, and thrombin was removed by adsorption to benzamidine-Sepharose 6B (22). Activity of the 7-kDa HRG-β3 EGF-like domain was determined by the ability to induce tyrosine phosphorylation of full-length ErbB-3 as well as ErbB-4 molecules, recombinantly expressed in NIH-3T3 cells. Monoclonal antibody to the extracellular domain of the human EGF receptor was obtained from Upstate Biotechnology, Inc. Rabbit polyclonal antibody to the extracellular domain of the human EGF receptor (23), α-adaptin (15), and the M6 antibody to the carboxyl-terminal domain of human ErbB-2 (24) were described previously. ErbB-3-specific peptide antisera have been characterized previously (25). ErbB-4 antisera was raised against a bacterially expressed GST fusion protein containing amino acids 1108–1264 (5) of the ErbB-4 carboxyl terminus. Polyclonal antibody to Shc was purchased from Transduction Laboratories, Inc. 35S-labeled amino acids (Trans35S-label) were obtained from ICN. [3H]Thymidine was purchased from DuPont NEN. Heregulin β1 and 125I-heregulin β1 were generous gifts from Dr. Mark Sliwkowski (Genentech).

Cell Culture and Chimeric Receptor Constructs—NIH-3T3 fibroblasts expressing human EGF receptor/ErbB chimeras or wild-type human ErbB receptors were used. All cells were routinely grown in Dulbecco’s Modified Medium of DMEM supplemented with 20 mM Hepes, pH 7.4, 10% calf serum, and 50 μM gentamicin. EGFR/ErbB-2 was composed of the extracellular domain of the human EGF receptor and the transmembrane and intracellular domains of the human ErbB-2 (17). ErbB-3 and ErbB-4 chimeric receptors were composed of the extracellular and transmembrane domains of the EGFR and the intracellular domains of the human ErbB-3 and ErbB-4, respectively. Transfectants expressing chimeric EGFR/ErbB-2 (26) and EGFR/ErbB-3 (25, 27) have been described and characterized elsewhere. The chimeric EGFR/ErbB-4 receptor was engineered by joining the EGF receptor extracellular and transmembrane domains (corresponding to amino acid position 24–658 in Ref. 28) with the ErbB-4 cytoplasmic domain (corresponding to amino acid position 690–1308 in Ref. 5). To facilitate its construction, a unique restriction endonuclease site was introduced into the full-length ErbB-4 coding sequence by recombinant PCR at a position corresponding to nucleotides 2039–2098 (5). To obtain long terminal repeat EGF/ErbB-4, the 1.9-kilobase pair Sall fragment of the ErbB-4 cytoplasmic domain was substituted for the 2.1-kilobase pair Sall fragment containing the ErbB-3 cytoplasmic domain in the long terminal repeat EGF/ErbB-3 expression vector (25). The resulting construct was characterized by restriction mapping and nucleotide sequence analysis of the junctions and PCR-amplified regions. A full-length ErbB-4 expression vector under long terminal repeat transcriptional control was generated by cloning the ErbB-4 open reading frame (29) in the unique SalI site of Z1Pneo (23) to obtain long terminal repeat ErbB-4. Expression vectors were transfected into NIH-3T3 cells by standard calcium phosphate precipitation.

As determined by 125I-EGF binding assays at 5°C (in binding medium of DMEM supplemented with 20 mM Hepes, pH 7.4, and 0.1% bovine serum albumin), the average number of receptors/cell was approximately 0.7–2.0 × 10⁶ for the wild-type EGF receptor, 2–6 × 10⁶ for EGF/ErbB-2, 0.6–1.5 × 10⁶ for EGF/ErbB-3, and 0.8–4.5 × 10⁶ for EGF/ErbB-4. Similar assays indicated the presence of approximately 1.2 × 10⁶ 125I-heregulin binding sites/cell in cells expressing the wild-type ErbB-4 receptor. In all cell lines, human EGFR/ErbB molecules or wild-type ErbB molecules are over-expressed by more than 100-fold compared with the endogenous levels of mouse ErbB or EGF receptors.

Internalization of 125I-EGF and 125I-Herregulin—To monitor ligand internalization, a previously described (17) protocol was followed. In brief, cells cultured in 12-well dishes were incubated with 125I-EGF (2 ng/ml) or 125I-heregulin β1 (2 ng/ml) at 37°C for 1–6 min. At the indicated times, the medium was aspirated and cells were rapidly washed with ice-cold DMEM. Subsequently, surface-bound 125I-growth factor was removed by a rapid acid (pH 2.8) wash and quantitated as a measure of surface-bound 125I-ligand. The cells were then solubilized in 1 M sodium hydroxide to quantitate internalized radioactivity. Non-specific binding, measured in the presence of a 200-fold excess of unlabeled ligand, represented not more than 10% of the total cell associated radioactivity. Specific binding is reported for all data points.

Receptor Down-regulation and Degradation—Ligand-induced receptor down-regulation was measured as described previously (17). In brief, cells grown in six-well dishes were incubated at 37°C for 0–2 h without or with EGF (200 ng/ml) in binding medium (DMEM plus 20% FCS, pH 7.3, and 0.1% bovine serum albumin) and then rinsed with cold DMEM. Surface-bound growth factor was removed by a pH 4.5 acid wash for 2 min at 4°C. The number of ligand binding sites on the cell surface was then determined by incubating cells with 125I-EGF (100 ng/ml) at 4°C for at least 1 h.

The degradation of metabolically labeled receptors in the absence or presence of EGF or heregulin was measured as described previously (17, 30). Cells were radiolabeled by incubation with 35S-labeled amino acids overnight at 37°C and subsequently washed with DMEM to remove unincorporated radiolabeled amino acids. The monolayers were then incubated at 37°C without or with EGF (300 ng/ml) or heregulin (100 ng/ml) in binding medium for 0.2, and 6 h before solubilization in TGH buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.2) plus 100 mM NaCl and protease and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 544 μM iodoacetamide). Receptors were immunoprecipitated with polyclonal antibodies as described below and isolated on SDS gels. The amount of radioactivity in bands corresponding to the receptor were quantitated using a PhosphorimagereM (Molecular Dynamics).

Immunoprecipitation and Immunoblotting—After growth factor treatment, cultures were washed with ice-cold Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) and solubilized at 4°C in the TGH/NaCl/protease and phosphatase inhibitors buffer described above. For the immunoprecipitation of receptor complexes with AP-2, NaCl was omitted from the solubilization buffer (13). Lysates were clarified by centrifugation, 15,000 × g, 10 min at 4°C. Immunoprecipitation of receptors or AP-2 complexes was accomplished by a 2-h incubation at 4°C with primary antibody and then a 1-h incubation with protein A-Sepharose CI-4B (Sigma) or protein G-Sepharose 4B beads (Zymed, Inc.) for polyclonal or monoclonal antibody, respectively. Immunocomplexes were washed 3 times with TGH/NaCl buffer in Laemmli buffer (31), and boiled. The proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5%) and transferred to nitrocellulose membranes. The membranes were blocked by incubation for 1 h at room temperature with TBST (0.05% Tween 20, 150 mM NaCl, 50 mM Tris, pH 7.5) plus 3% bovine serum albumin prior to incubation with the indicated blotting antibody for 2 h at room temperature. After washing the membranes with TBST, blots were incubated for 1 h with protein A-conjugated horseradish peroxidase (Zymed, Inc.) or 125I-protein A (ICN), washed for 30 min with TBST, and visualized, respectively, by enhanced chemiluminescence (DuPont) or autoradiography using Kodak X-Omat AR film.

**GST Fusion Proteins**—GST fusion proteins containing the carboxyl terminus of either the EGF receptor (residues 945–1186) or ErbB-4 (residues 989-1264) were obtained by bacterial expression. The GST-EGF fusion protein was obtained by recombinant PCR of the appropriate fragment from the human EGF receptor cDNA (28) followed by cloning in the pGEX-KT expression vector, between BamHI and EcoRI sites. GST sequences used in the PCR reaction were 5′-ATGGATCTCCTCAAAAAAGGCC CGGAGACCCC (upstream) and 5′-AGTAAATTCCTGCTCCTAAATCTCAGTGC (downstream). The ErbB-4 carboxyl-terminal coding sequence for amino acid residues 989-1264 (5) was PCR-amplified with a BamHI site 5′ and an EcoRI site 3′, using human ErbB-4 cDNA (29) as a template. The ErbB-4 carboxyl-terminal coding sequence was then directionally cloned into the BamHI and EcoRI I sites of pGEX-2T (Pharmacia). Bacterial expression and GST fusion protein purification followed a standard protocol. Briefly, exponential bacterial cultures were grown in LB media containing a second antibiotic, with IPTG induction at 4°C and subsequent purification on glutathione-Sepharose. The fusion proteins were then used for GST pull-down experiments or further purification on glutathione-Sepharose beads. The purified GST fusion proteins were used as a template for GST pulldown, followed by immunoprecipitation with AP2, polyclonal or monoclonal antibodies, respectively.
Growing at 37°C in LB/ampicillin medium were induced for 3 h by the addition of 0.5 ml isopropyl-1-thio-β-D-galactopyranoside. The bacteria were then lysed in an ice-cold PBS supplemented with 1% Triton X-100, 100 μg/ml leupeptin, 100 μg/ml aprotime, 0.2 M phenylmethylsulfonyl fluoride, and 2 mM EDTA. After sonication and centrifugation (25,000 × g, 30 min) of the lysates, samples were incubated with glutathione-agarose beads (Sigma), and the beads were then washed with a solution composed of PBS and 1% Triton X-100 and then washed with PBS. The amount of GST fusion protein bound to the agarose beads was quantified by adding a small amount to SDS sample buffer, along with bovine serum albumin standards (Sigma), to Coomassie-stained SDS-polyacrylamide gels to approximate the amount of fusion protein. Typically, the concentration obtained was 1–2 μg of fusion protein/ml of beads.

For adaptin binding experiments, GST fusion proteins bound to glutathione-agarose beads were washed 3 times in cold TGH/NaCl buffer. Lysates from NIH-3T3 murine fibroblasts, prepared in TGH/NaCl buffer as described above for the immunoprecipitation protocol, were incubated for 1.5 h at 4°C with the GST fusion proteins. The ratio of protein lysate to GST fusion protein was approximately 1000:1. After the incubation, the beads were washed 5 times with TGH/NaCl and processed for immunoblotting of AP-2 as described above.

RESULTS

EGF-dependent Tyrosine Kinase Activity of Chimeric Receptors—Receptor autophosphorylation is necessary for EGF-induced endocytosis of the EGF receptor, including AP-2 association (15, 16), 125I-EGF internalization (11), and receptor down-regulation and degradation (11). Therefore, the tyrosine kinase activity of chimeric receptors in response to EGF binding was assayed. After EGF addition to intact cells expressing various receptor constructs, individual receptors were immunoprecipitated, and receptor autophosphorylation was assayed by Western blotting with anti-phosphotyrosine (Fig. 1A). Also, unfraccionated lysates from each cell line were electrophoresed and blotted with anti-phosphotyrosine to assess the EGF-dependent phosphorylation of total cellular proteins (Fig. 1B). The data show that all receptors responded to the addition of EGF with increased levels of receptor autophosphorylation and cellular protein phosphorylation. Consistent with published data, the basal level of phosphotyrosine was low in the EGFR and EGFR/ErbB-3 molecules, somewhat higher with EGFR/ErbB-4, and high with EGFR/ErbB-2. These results demonstrate that the chimeric receptors are EGF-dependent tyrosine kinases. Previously we have shown that the EGFR/ErbB-2 (32, 33) and EGFR/ErbB-3 (25, 27) chimeric receptors mediate biological responses, such as mitogenesis, in response to EGF. As judged by EGF-induced [3H]thymidine incorporation, the EGF/ErB-4 molecule described herein also mediates a mitogenic response to EGF (data not shown).

Internalization of Chimeric ErbB Receptors—EGF binding to its receptor induces the rapid internalization of ligand-receptor complexes, particularly at low surface receptor occupancy that minimizes receptor recycling (11). A previous study (17) showed that chimeric EGFR/ErbB-2 molecules, however, do not mediate the rapid internalization of 125I-EGF. We have, therefore, measured the capacity of all ErbB family members to internalize 125I-EGF. The data, shown in Fig. 2, demonstrate that 125I-EGF is rapidly internalized by EGFR receptors, but internalization mediated by the ErbB-2, ErbB-3, or ErbB-4 cytoplasmic domains is extremely low. The 125I-EGF internalization rate for chimeric receptors (k_1, 0.04) is 3-fold lower than the internalization rate exhibited by the EGF receptor (k_1, 0.13).

Recent studies (13, 15, 16) have shown that the EGF receptor associates with the plasma membrane coated-pit adaptor complex, AP-2, which may facilitate internalization of occupied receptors. This association is increased when cells are incubated with EGF at 37°C, but is not enhanced following ligand binding at 4°C (13). We have, therefore, determined whether

![Figure 1](http://www.jbc.org/content/5253/17872/F1)

**Fig. 1.** EGF-dependent activation of chimeric receptors. Cells expressing EGF receptors or EGF/Erb receptors were incubated overnight in medium containing 0.5% calf serum and then incubated for 1 h at 4°C without or with EGF (100 ng/ml). Panel A, after two washes with cold Ca^2+,-Mg^2+-free PBS, cells were lysed, and an aliquot (1 mg) of each lysate was then used to assay receptor autophosphorylation by receptor immunoprecipitation and anti-phosphotyrosine Western blotting, as described under “Experimental Procedures.” Panel B, a second aliquot (100 μg) of each lysate was directly electrophoresed and Western blotted with anti-phosphotyrosine to detect tyrosine phosphorylated cellular proteins.

The impaired EGF internalization by ErbB-2, ErbB-3, and ErbB-4 chimeric receptors is paralleled by deficient association with AP-2. Cells expressing the EGF receptor or the indicated chimeric receptors were incubated with EGF at 4°C or 37°C, and their association with AP-2 was measured. As a control, association of each receptor with the tyrosine kinase substrate Shc was also assayed. Unlike AP-2, Shc contains specific phosphotyrosine recognition sequences, both SH2 (34) and PTB domains (35–37), and its association with activated receptors is temperature-independent (38). The cells were also incubated in a K^+–free media, which impairs coated-pit formation and endocytosis and, thereby, enhances the accumulation of AP-2-receptor complexes (33). The data presented in Fig. 3 show that while the initially basal level (i.e. 4°C) of α-adaptin present in all receptor immunoprecipitates, enhanced adaptin-receptor
Endocytosis of ErbB Receptors

To substantiate the internalization of receptors—In the presence of EGF, most internalized EGF receptors are ultimately degraded in lysosomes (26), which substantially alters the metabolic half-life of the entire cellular receptor population (22). To substantiate the internalization data, we have metabolically labeled receptor molecules with $^35$S-labeled amino acids and used a pulse-chase protocol to measure receptor half-lives in the absence or presence of EGF (Table I). In the NIH-3T3 cell background, the half-life of the EGF receptor was decreased approximately 3–4-fold in the presence of EGF. The half-lives of the EGFR/ErbB-2 and EGFR/ErbB-4 receptors were, in the absence of EGF, similar to that of the EGF receptor; however, the presence of EGF did not substantially alter these half-life values. Compared with the other receptors, the EGFR/ErbB-3 receptor displayed a much shorter half-life in the absence of EGF. However, its metabolic half-life was not significantly changed by the presence of EGF. These results indicate that all ErbB family members, only the occupied EGF receptor is rapidly internalized and degraded.

The net result of ligand-induced receptor internalization and degradation is a decrease in the number of functional receptors on the cell surface, a phenomenon termed down-regulation. Down-regulation, which may have a role in regulating the level of growth factor signaling, is also influenced by other factors, such as the relative rates of receptor synthesis and receptor recycling. Therefore, the capacity of EGF to down-regulate various chimeric ErbB family members was measured. Cells were incubated with unlabeled EGF for varying periods of time and subsequently washed to remove unbound ligand and surface-associated EGF. Then the capacity of the cells to bind $^{125}$I-EGF was assayed at 4°C. As shown in Fig. 4, EGF receptors were down-regulated by approximately 65%, while down-regulation of the EGFR/ErbB-2 and EGFR/ErbB-4 receptors was negligible, about 15%. Under these conditions, the EGFR/ErbB-3 receptor exhibited an intermediate level of down-regulation, approximately 40%. The mechanism of the apparent EGFR/ErbB-3 down-regulation is unclear, but it may reflect its short half-life compared with other ErbB family members.

Analysis of the Native ErbB-4 Receptor—The preceding experiments indicate that, within the context of chimeric receptors, the activated ErbB-2, ErbB-3, or ErbB-4 cytoplasmic domains do not facilitate rapid ligand internalization, AP-2 association, receptor degradation, or receptor down-regulation. While these ErbB cytoplasmic domains are tyrosine phosphorylated in response to heterologous ligand binding and the chimeric receptors do transduce biological responses, such as mitogenesis, it may be argued that receptor trafficking functions are not accurately reproduced in the chimeric molecules. Therefore, cells transfected with the native ErbB-4 molecule, which is a high affinity receptor for heregulin (9), have been utilized to measure $^{125}$I-heregulin internalization and the capacity of heregulin to alter the metabolic half-life of ErbB-4 or to induce AP-2 association with the native ErbB-4 receptor.

![Figure 2](http://example.com/fig2.png)

**Fig. 2.** $^{125}$I-EGF internalization by chimeric receptors. Cells expressing EGF receptors or EGFR/ErbB receptors were incubated for 1–6 min at 37 °C with $^{125}$I-EGF (~2 ng/ml). At the indicated times, the monolayers were washed with cold DMEM to remove unbound $^{125}$I-EGF and then acid-washed to remove surface bound $^{125}$I-EGF, as described under "Experimental Procedures." Radioactivity present in the acid washes was quantitated as surface-associated $^{125}$I-EGF. The remaining cell-associated, or internalized, radioactivity was quantitated following solubilization of the cells. To compare the internalization capacity of different cell lines, data at each time point are expressed as the ratio of internalized to surface radioactivity.

**Fig. 3.** Analysis of α-adaptin association with chimeric receptors. Cells expressing EGF or EGFR/ErbB receptors were placed in potassium-depleted media (13) and then incubated at 4 °C for 45 min with EGF (100 ng/ml). To induce maximal AP-2 association, the cultures were then shifted to 37 °C and incubated for 10 min, while control cultures were maintained at 4 °C. Cell lysis, receptor immunoprecipitation, and Western blotting for α-adaptin, Shc, and receptors were performed as described under "Experimental Procedures." association at 37 °C was only detected with the EGF receptor. Western blots show that the level of receptors in all immunoprecipitates was not dramatically different and that all receptors showed substantial levels of co-immunoprecipitated Shc. Interestingly, the p46 and p66 isoforms of Shc varied with different ErbB family members, but the p52 isoform associated with all ErbB chimeric molecules.

**Table I**

| Receptor     | Receptor half-life |
|--------------|--------------------|
|              | -EGF | +EGF |
| EGFR         | 6.5  | 1.5  |
| EGFR/ErbB-2  | 8.0  | 7.0  |
| EGFR/ErbB-3  | 3.5  | 3.0  |
| EGFR/ErbB-4  | 6.0  | 5.5  |

| Receptor | Receptor half-life |
|----------|--------------------|
|          | -EGF | +EGF |
| EGFR     | 6.5  | 1.5  |
| EGFR/ErbB-2 | 8.0  | 7.0  |
| EGFR/ErbB-3 | 3.5  | 3.0  |
| EGFR/ErbB-4 | 6.0  | 5.5  |

**Metabolic half-life of chimeric receptors in the absence and presence of EGF**

Cells expressing EGF receptors or EGFR/ErbB receptors were labeled with $^{35}$S-labeled amino acids for approximately 14 h, washed, and then incubated without or with EGF (300 ng/ml) for various periods of time at 37 °C. At each time point, the cells were lysed, and the receptors were immunoprecipitated, as described under "Experimental Procedures." The level of radioactivity in each receptor band was quantitated using a PhosphorImager, and the half-lives were calculated and rounded to the nearest 0.5 h.
The phenomenon of receptor-mediated internalization of growth factors was first described for EGF and its receptor (20), and the basic observation has since been established for a wide variety of growth factors and their receptors (11). Hence, it was unexpected that within the EGF receptor family, only the EGF receptor would mediate rapid ligand internalization and cleavage of a small fragment of the ErbB-4 molecule is also not induced by heregulin binding.

Finally, we examined the capacity of heregulin to induce the autophosphorylation of ErbB-4 and to stimulate the association of AP-2 with the ErbB-4 receptor (Fig. 6). These data show that while EGF did induce association of AP-2 with the EGF receptor at 37°C, heregulin failed to significantly stimulate ErbB-4 autophosphorylation at 37°C (Fig. 6A). Since AP-2 association in vivo requires receptor autophosphorylation (15, 16), the capacity of heregulin to induce ErbB-4 autophosphorylation was examined. The data in Fig. 6B demonstrate that heregulin did significantly stimulate ErbB-4 autophosphorylation. Similar levels of heregulin-induced ErbB-4 autophosphorylation were induced at 37 (10 min) or 4°C (1 h). We also tested AP-2 association with ErbB-4 compared with the EGF receptor in an in vitro system, employing GST fusion proteins containing either the carboxyl terminus of the EGF receptor or the carboxyl terminus of ErbB-4 (Fig. 7). Consistent with the results of Nesterov et al. (16), we obtained a significant level of AP-2 association with the EGF receptor-GST fusion protein. However, association of AP-2 with the ErbB-4-GST fusion protein was not detectable.

The receptor system described above offers the opportunity to ask whether a receptor that does not undergo ligand-induced internalization and down-regulation may be more mitogenic than a similar receptor, which is rapidly down-regulated, particularly at low growth factor concentrations. Therefore, we have used NIH-3T3 cells expressing EGF receptors or ErbB-4 receptors to assay the mitogenicity of increasing concentrations of EGF or heregulin (Fig. 8). The data indicate that for this comparison there is no substantial difference in the capacity of activated EGF and ErbB-4 receptors to elicit increased DNA synthesis in quiescent cells.

DISCUSSION

The receptor system described above offers the opportunity to ask whether a receptor that does not undergo ligand-induced internalization and down-regulation may be more mitogenic than a similar receptor, which is rapidly down-regulated, particularly at low growth factor concentrations. Therefore, we have used NIH-3T3 cells expressing EGF receptors or ErbB-4 receptors to assay the mitogenicity of increasing concentrations of EGF or heregulin (Fig. 8). The data indicate that for this comparison there is no substantial difference in the capacity of activated EGF and ErbB-4 receptors to elicit increased DNA synthesis in quiescent cells.

FIG. 4. EGF-induced down-regulation of chimeric receptors. Cells expressing EGF or EGF/ErbB receptors were incubated without or with EGF (300 ng/ml) for the indicated times at 37°C. Thereafter, the monolayers were rinsed with cold DMEM, and surface-bound EGF was removed by cold acid washes followed by two rinses with DMEM. The number of the EGF binding sites on the cell surface was then determined by incubating the cells with 125I-EGF (100 ng/ml) at 4°C for at least 1 h. Data are expressed as the percentage of 125I-EGF binding capacity relative to cells not exposed to unlabeled EGF.

FIG. 5. 125I-Heregulin internalization by wild-type ErbB-4. Cells expressing ErbB-4 or EGF receptors were incubated for 1–10 min at 37°C with approximately 2 ng/ml of either 125I-heregulin or 125I-EGF, respectively. Internalized and surface-bound radioactivity for each time point were assayed and plotted as described in Fig. 2.
growth factor-induced receptor down-regulation. The data of Sorkin et al. (17) did show that the chimeric EGFR/ErbB-2 is deficient in receptor-mediated endocytosis and that the carboxyterminus of the receptor determines the endocytic capacity. As the chimeric ErbB-2 receptor failed to mediate rapid ligand internalization in either NIH-3T3 or the human mammary adenocarcinoma cell line MDA-MD-134, it seems unlikely that cell background defines internalization capacity. However, no ligand for ErbB-2 has been identified and, therefore, the conclusions are based on the behavior of chimeric EGFR/ErbB-2 molecules. No data have been reported for the ErbB-3 and ErbB-4 molecules. The data in this manuscript show that chimeric EGFR/ErbB-3 and EGFR/ErbB-4 molecules fail to exhibit receptor-mediated endocytosis of EGF and that the wild-type ErbB-4 receptor fails to mediate heregulin internalization or heregulin-dependent receptor trafficking, such as enhanced metabolic degradation. Therefore, the analysis of ErbB-4 has utilized a chimeric molecule, which responds to EGF, as well as the wild-type receptor. The results with both molecules are consistent; the activated ErbB-4 cytoplasmic domain fails to mediate the rapid ligand-dependent receptor trafficking demonstrated by the EGF receptor. Using the data in Figs. 2 and 5, we calculate that the $^{125}$I-EGF internalization rate constant, $k_e$, is approximately 0.13 for the wild-type EGF receptor, approximately 0.04 for the chimeric ErbB-2, ErbB-3, ErbB-4 receptors, and about 0.07 for the native ErbB-4 medi-
ated internalization of 125I-labeled heregulin. These low internalization rate constants for ErbB-2, -3, -4 are equivalent to our published internalization rate of the kinase-negative EGF receptor (39) and imply that only a constitutive basal level of receptor endocytosis is operative for the ErbB-2, -3, -4 molecules.

The analysis of ErbB-3 relies on data from chimeric molecules, as does the previously published analysis (17) of ErbB-2. Heterodimeric complexes of ErbB-3 and ErbB-2 are reported to form a high affinity receptor for heregulin (10) and to be the basis of co-operative signaling and neoplasic transformation by these two ErbB family members (10, 40). This raises the possibility that a heterodimer of ErbB-2 and ErbB-3 might mediate rapid heregulin internalization. However, mammary tumor lines that express high levels of ErbB-2 and ErbB-3, relative to ErbB-4, also demonstrate a low rate of 125I-labeled heregulin internalization. In the NIH-3T3 cells employed in this study, each receptor has been over-expressed in a cell line in which the endogenous levels of EGF receptor, ErbB-2, ErbB-3, and ErbB-4 are relatively low. The overexpression of each transfected receptor is approximately 100-fold relative to the level of endogenous ErbB molecules. Therefore, it seems unlikely that significant levels of heterodimerization between ErbB family members complicate our analyses of ligand binding.

While defined internalization codes have been identified in certain non-growth factor receptors, such as the low density lipoprotein, mannose 6-phosphate, and transferrin receptors (41), internalization codes have not been clearly identified in any growth factor receptor. Using deletion analysis, Chang et al. (42) came to the conclusion that the ligand internalization capacity of the EGF receptor is dependent on multiple or functionally redundant internalization codes. Primarily identified were sequences 996FYRSL and 973QQGFF sequences within the cytoplasmic domain of the EGF receptor. Neither of these putative internalization codes, however, is preserved in the cytoplasmic domains of ErbB-3 and ErbB-4. The cytoplasmic domain of ErbB-2 does contain FYRSL and QQGFF sequences at approximately the same relative position as the proposed internalization codes in the EGF receptor. However, the only studies reporting ErbB-2 internalization are experiments in which antibodies to the ErbB-2 extracellular domain have been used to stimulate the internalization process (18, 44, 45). It is unclear and perhaps unlikely that antibody-induced internalization is analogous to the rapid internalization process promoted by growth factors. Efficient EGF receptor internalization and down-regulation are also thought to involve the phosphorylation of Ser1046 and Ser1047 in the receptor carboxyl terminus (43). While Ser1046 is retained in ErbB-2 and -4, Ser1047 is not retained in ErbB-2, -3, or -4.

The mechanisms of receptor-mediated internalization of growth factors are not understood, but recent data suggest that the cationic coated pit adapter protein AP-2 might mediate activated EGF receptor association with coated pits and hence internalization (13, 15, 16). If the association of AP-2 and the EGF receptor is functionally related to the internalization process, then a testable prediction would be that receptors that do not undergo rapid internalization should not form complexes with AP-2. The data in this manuscript show that this is not the case for all ErbB molecules that are internalization impaired.

Acknowledgments—The authors appreciate the excellent technical assistance of Sandra Ermini and Usha Barad and manuscript preparation by Sue Carpenter and Susan Heaver. The generous gift of 125I-labeled heregulin from Dr. Mark Slwikowski (Genentech) is gratefully acknowledged.
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J. Biol. Chem. 1996, 271:5251-5257.
doi: 10.1074/jbc.271.9.5251

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