Epidermal Growth Factor (EGF) Modulation of Feline Sarcoma Virus *fms* Tyrosine Kinase Activity, Internalization, Degradation, and Transforming Potential in an EGF Receptor/*v-fms* Chimera

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The feline sarcoma virus oncogene *v-fms* has significantly contributed to the dissection of peptide growth factor action since it encodes the transmembrane tyrosine kinase gp140^{*v-fms*}, a transforming version of colony-stimulating factor 1 receptor, a member of the growth factor receptor tyrosine kinase family. In this study, the functional significance of structural differences between distinct tyrosine kinase types, in particular between cellular receptors and viral transforming proteins of distinct structural types, has been further investigated, and their functional compatibility has been addressed. For this purpose, major functional domains of three structurally distinct tyrosine kinases were combined into two chimeric receptors. The cytoplasmic gp140^{*v-fms*} kinase domain and the kinase domain of Rous sarcoma virus pp60^{*v-src*} were each fused to the extracellular ligand-binding domain of the epidermal growth factor (EGF) receptor to create chimeras EFR and ESR, respectively, which were studied upon stable expression in NIH 3T3 fibroblasts. Both chimeras were faithfully synthesized and routed to the cell surface, where they displayed EGF-specific, low-affinity ligand-binding domains in contrast to the high- and low-affinity EGF-binding sites of normal EGF receptors. While the EFR kinase was EGF controlled for autophosphorylation and substrate phosphorylation *in vitro*, *in vivo*, and in digitonin-treated cells, the ESR kinase was not responsive to EGF. While ESR appeared to recycle to the cell surface upon endocytosis, EGF induced efficient EFR internalization and degradation, and phorbol esters stimulated protein kinase C-mediated downmodulation of EFR. Despite its ligand-inducible kinase activity, EFR was partly EGF independent in mediating mitogenesis and cell transformation, while ESR appeared biologically inactive.

The oncogenes of tumor viruses have critically contributed to our understanding of the regulation of cell proliferation, since their expression products often encode altered key elements in cell growth control. These include peptide hormones, their cell surface receptors, other protein kinases, GTP-binding proteins, and other proteins including nuclear factors which participate in signaling cascades that connect the cell surface to the genome in the cell nucleus (4, 55). Peptide growth factors are important components of a cellular communication network that governs proliferation, differentiation, development, and metabolism of multicellular organisms. At their target cells, they bind to specific cell surface receptors that translate this interaction into an intracellular signal. The best characterized of these receptors belong to a family of structurally related molecules that share a tyrosine-specific protein kinase activity, which is essential for their physiological signaling function (54). Yet the mechanisms that convert an extracellular interaction between ligand and receptor into an intracellular signal and subsequently into a specific cellular response are still poorly understood. The receptor tyrosine kinases characterized to date share a three-domain structure of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic domain with extensive amino acid sequence homology to other protein kinases, and several

subclasses have been defined on the basis of distinct structural features (53). Representatives such as the epidermal growth factor (EGF) receptor (52) and the insulin receptor (14, 51) display a monomeric versus a heterotetrameric structure but share cysteine-rich clusters in their extracellular domains and a continuous tyrosine kinase domain. In contrast, the colony-stimulating factor 1 (CSF-1) receptor/gp165^{*c-fms*} (5, 47) and its oncogenic counterpart, the transforming protein gp140^{*v-fms*} of feline sarcoma virus (FeSV) (20), display 12 distinct single cysteine residues in their extracellular domains and an insertion sequence in their tyrosine kinase domains.

In this study, the functional significance of structural differences between distinct tyrosine kinase types, in particular between cellular receptors and transforming proteins of distinct structural types, has been further investigated, and their functional compatibility has been addressed. For this purpose, major functional domains of three structurally distinct tyrosine kinases were combined into two chimeras. In particular, I have addressed the question whether the cytoplasmic domain of one of the cell-transforming members of the platelet-derived growth factor receptor superfamily, the gp140^{*v-fms*} tyrosine kinase, can be controlled by the ligand-binding domain of the EGF receptor via the transmembrane domain of gp140^{*v-fms*}. Since gp140^{*v-fms*} and the EGF receptor display 12 single cysteine residues versus two cysteine-rich clusters in their respective extracellular domains, I have also investigated whether a chimera (EFR) which combines one of the cysteines of gp140^{*v-fms*} with the cysteine-rich clusters of the EGF recep-

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tor would be functional. In this way, the functional compatibility of the two structurally distinct extracellular receptor domains for the specific binding of ligand has been tested by joining extracellular sequences from both receptor types. Consequently, EFR is composed of most of the ligand-binding domain of the EGF receptor fused to sequences of the FeSV transforming protein gp140^{v-fms} that include an extracellular segment containing one cysteine and the complete transmembrane and cytoplasmic domains.

A number of receptor chimeras have been investigated before. These studies suggest that many combinations of major structural domains of distinct receptor tyrosine kinase types are functional (36, 37, 54). To test a combination of transmembrane receptor tyrosine kinase with distinct nonreceptor, non-transmembrane tyrosine kinase sequences, a chimera was created, consisting of EGF receptor extracellular, transmembrane, and juxtamembrane sequences fused to the kinase domain of pp60^{v-src}, the transforming protein of Rous sarcoma virus, which is a structurally distinct membrane-associated tyrosine kinase that does not span the plasma membrane (7). In this chimera, ESR, the EGF receptor kinase domain had been replaced by the pp60^{v-src} kinase at a similar distance from the EGF receptor transmembrane domain to test whether this construct would still result in a ligand-responsive tyrosine kinase despite the combination of domains of clearly distinct proteins and despite the lack of the amino-terminal sequence of pp60^{v-src}, which has no counterpart in the EGF receptor sequence (7, 52). Consequently, in the chimeras EFR and ESR tyrosine kinase domains of distinct types of viral oncogene products were combined with EGF receptor ligand-binding sequences. Upon expression in NIH 3T3 fibroblasts, the functional consequences of heterologous domains within a membrane-spanning cell surface receptor were studied.

The results indicate that both chimeras were faithfully synthesized and routed to the cell surface, where they displayed EGF-specific, low-affinity ligand-binding domains in contrast to the high- and low-affinity EGF-binding sites of normal EGF receptors. While the EFR kinase was EGF controlled for autophosphorylation and substrate phosphorylation in vitro, in vivo, and in digitonin-treated cells, the ESR kinase was not responsive to EGF. While ESR appeared to recycle to the cell surface upon endocytosis, EGF induced efficient EFR internalization and degradation, and phorbol esters stimulated protein kinase C (PKC)-mediated downmodulation of EFR. Despite its ligand-inducible kinase activity, EFR was partly EGF independent in mediating mitogenesis and cell transformation, while ESR appeared biologically inactive.

MATERIALS AND METHODS

Reagents. Receptor-grade EGF from mouse submaxillary glands was obtained from Collaborative Research, Inc.; ¹²⁵I-labeled mouse EGF (37 TBq/mmol), [γ -³²P]ATP (>185 TBq/mmol), L-[³⁵S]methionine (47 TBq/mmol), and [methyl-³H]thymidine (3 TBq/mmol) were obtained from Amersham, Inc.; protein A-Sepharose (CL-4B) was obtained from Pharmacia, Inc.; all cell culture reagents including Geneticin (G418 sulfate) were obtained from GIBCO Laboratories; transferrin, tetradecanoyl-phorbol-acetate (TPA), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), aprotinin, and pepstatin were obtained from Sigma Chemical Company; Bio-Gel A-50m agarose was obtained from Bio-Rad Laboratories; agar (Noble) was obtained from Difco Laboratories; and En³Hance and Aquasol scintillation cocktail were obtained from New England Nuclear.

The mouse monoclonal antibodies MAbRI (58) and MAb108, which specifically recognize the extracellular domain of the human EGF receptor, the mouse monoclonal antibody 5E2 directed against phosphotyrosine amino acid residues, and a polyclonal rabbit antiserum against Rous sarcoma virus pp60^{v-src} (31) were employed in various experiments.

Cell culture. Mouse NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1 g of glucose per liter, 2 mM L-glutamine, penicillin, and streptomycin. Cells were cultured at 37°C with 7.5% CO₂ and were passaged once a week.

Expression plasmids. All cDNA constructs were introduced into an expression plasmid (37) containing simian virus 40 early promoter elements and 3'-untranslated sequences of the hepatitis virus surface antigen gene for receptor cDNA expression (6), for the expression of a dihydrofolate reductase (DHFR) cDNA (49), and for a cDNA conferring resistance to the aminoglycoside antibiotic G418 (*neo*). Sequences of the bacterial plasmid pBR322 allowed replication and selection (ampicillin) in *Escherichia coli*. The creation of the human EGF receptor (HER) expression plasmid has been described elsewhere (38).

HER and FeSV-*fms* (20) coding DNA sequences were combined at *NaeI* and *NdeI* sites, respectively, to create the EFR expression construct by joining a 1.9-kb *SstI*-*NaeI* fragment of HER and a 2-kb *NdeI*-*BamHI* fragment of *v-fms* subcloned in pBR322 with a double-stranded synthetic oligonucleotide (5'-GGCATGCGCGCA-3' and 3'-CCGTACGCGCGTAT-5'). The created 3.9-kb *SstI*-*BamHI* fragment was end filled at the *BamHI* site, fused with a 2.7-kb *SstI*-*SmaI* fragment of pUC13, and amplified in *E. coli*. A 3.9-kb *SstI* (partially cleaved)-*HindIII* (end filled) fragment of the complete chimeric EFR coding sequence was joined with an *SstI*-*EcoRV* fragment containing the control elements of the expression plasmid including DHFR cDNA and sequences conferring resistance to the aminoglycoside antibiotic G418 (37).

HER and Rous sarcoma virus *src* (strain Schmidt-Ruppin A) (7) coding cDNA sequences were combined at *EcoRI* and *HgaI* sites, respectively, to create the ESR expression construct by joining a 2.2-kb *SstI*-*EcoRI* (partially cleaved) fragment of HER and a 1.1-kb *HgaI*-*PvuI* fragment of *v-src* with the double-stranded synthetic oligonucleotide (5'-AATTCGATC TAG-3' and 3'-GCTAGATCTTTAG-5'). The created 3.3-kb *SstI*-*PvuI* fragment was converted to a blunt end at the *PvuI* site, fused with a 2.7-kb *SstI*-*SmaI* fragment of pUC13, and amplified in *E. coli*. A 3.3-kb *SstI*-*HindIII* (end filled) fragment of the complete chimeric ESR coding sequence was joined with an *SstI*-*EcoRV* fragment containing the control elements of the expression plasmid including DHFR cDNA and sequences conferring resistance to the aminoglycoside antibiotic G418 (37).

Expression plasmids and control plasmids lacking receptor sequences were amplified and purified from *E. coli* for transfection of mouse NIH 3T3 fibroblasts by the alkaline extraction procedure followed by gel filtration (A-50m agarose; Bio-Rad).

DNA transfection. Purified expression plasmids were introduced into mouse NIH 3T3 fibroblasts by calcium phosphate precipitation (19). Expressing cells were first selected with 400 μ g of G418 sulfate (actual drug) per ml, and expression was further enhanced by gene amplification in the presence of 1 μ M methotrexate in glycine-, hypoxanthine- and thymidine-free medium.

Metabolic labeling. Subconfluent cells grown in 8-cm-diameter culture dishes were washed twice with phosphate buffered

saline (PBS). Five milliliters of methionine-free cell culture medium prepared from a MEM select amine kit (GIBCO) including 10% fetal calf serum was added, and the incubation was continued for 20 h with 300 μ Ci of [35 S]methionine.

Cell lysis and immunoprecipitation. Cell monolayers were washed twice with PBS and solubilized in 0.7 ml of 1% Triton X-100 lysis buffer as described elsewhere (28). Three hundred-microliter aliquots were diluted fourfold with detergent-free lysis buffer and incubated with 1 μ l of MAb108 and 50 μ l of protein A-Sepharose slurry (50% [vol/vol]; prewashed in detergent-free lysis buffer) for 3 h at 4°C. The immunoprecipitates were washed four times in 0.9 ml of HNTG buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100).

In vitro autophosphorylation and gel electrophoresis. Detergent lysates of expressing cells (300- μ l aliquots) were incubated with 5 μ g of EGF per ml for 30 min at 4°C. Proteins were immunoprecipitated for 1 h at 4°C with MAb108, which had been prebound to prewashed protein A-Sepharose. The washed immunoprecipitates (volume, 25 μ l) were adjusted to 5 mM MnCl₂, 20 μ Ci of [γ - 32 P]ATP at 0.15 μ M (at 50 μ M for EFR) was added, and the mixture was incubated for 10 min at 0°C. The reaction was stopped by boiling with 50 μ l of 1.5 $\times$ concentrated sodium dodecyl sulfide (SDS) sample buffer. Proteins were analyzed on SDS-7% polyacrylamide gels. For autophosphorylation experiments, gels were fixed and dried immediately. To detect [35 S]methionine radioactivity, proteins were immunoprecipitated and directly loaded on gels. Gels were fixed for 1 h in 10% acetic acid-35% methanol, incubated for 1 h in En³Hance (New England Nuclear), precipitated for 1 h in H₂O, and dried under vacuum at 60°C.

In vivo protein phosphorylation. Subconfluent cells in 8-cm-diameter culture dishes were incubated in medium containing 0.5% fetal calf serum for 24 h, and cellular proteins were subsequently labeled with [γ - 32 P]ATP in the presence or absence of ligand after cell membranes had been partially permeabilized with the detergent digitonin. For this purpose, the culture medium was removed and cells were incubated in 0.7 ml of permeabilization buffer containing 75 μ g of digitonin per ml and 70 μ Ci of [γ - 32 P]ATP at 15 μ M (buffer A) (16) for 6 min at 21°C in the presence or absence of 160 nM EGF. The labeling solution was removed, and cells were solubilized for 5 min at 4°C in 0.7 ml of lysis buffer containing 1% Triton X-100, 20 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid], 20 μ M ZnCl₂, 10 mM NaF, 10 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% aprotinin, 2 mM PMSF, and 0.1 mM leupeptin. Half of the lysate was diluted fourfold with detergent-free lysis buffer, and proteins were immunoprecipitated with mouse monoclonal phosphotyrosine antibody 5E2 (3 μ l) or MAb108 (1 μ l) and 50 μ l of prewashed protein A-Sepharose slurry (50% [vol/vol]) for 2 h at 4°C. Precipitates were washed four times with 0.1% Triton X-100 lysis buffer, boiled in SDS sample buffer, and analyzed on an SDS-7% polyacrylamide gel or an SDS-7 to 15% polyacrylamide step gradient gel.

TPA modulation of ligand binding. Subconfluent cell monolayers in 2.2-cm-diameter wells were rinsed and incubated in 0.5 ml of DMEM binding medium containing 0.5% BSA and 20 mM HEPES (pH 7.3) in the presence or absence of 100 nM TPA for 75 min at 37°C with 7.5% CO₂. Cells were allowed to cool to 21°C, and 0.1 ml of 125 I-EGF was added at eight different final concentrations varying from 8 to 18,000 pM and with a constant specific activity of 33,000 cpm/ng for ligand binding at 21°C for 2 h. Background binding was determined in

the presence of a 100- to 1,000-fold excess of unlabeled ligand. Cells were rinsed three times with ice-cold PBS and solubilized in 0.2 N NaOH and 0.1% SDS, and the radioactivity was determined in a gamma-radiation counter. Scatchard plots were obtained with a computer program developed by S. Felder (Rorer Biotechnology, Inc., King of Prussia, Pa.).

Time dependence of ligand-binding modulation. Subconfluent cell monolayers in 2.2-cm-diameter wells were incubated in 0.5 ml of DMEM binding medium containing 0.5% BSA and 20 mM HEPES (pH 7.3) in the presence of 100 nM TPA for various times at 37°C with 7.5% CO₂. Cells were rinsed twice with ice-cold binding medium and were incubated with 0.4 fM of 125 I-EGF in 0.5 ml at 25 TBq/mmol for 15 h at 4°C. Background binding was determined in the presence of a 100- to 1,000-fold excess of unlabeled ligand. Cells were rinsed three times with ice-cold PBS and solubilized in 0.2 N NaOH and 0.1% SDS, and the radioactivity was determined in a gamma-radiation counter.

Ligand internalization. Subconfluent cell monolayers in 2.2-cm-diameter wells were rinsed twice with serum-free cell culture medium including 0.5% BSA and 20 mM HEPES (pH 7.3) and were incubated in the presence of 2 nM 125 I-EGF (6 TBq/mmol) in a 1-ml volume at 37°C with 7.5% CO₂ for various times. After three washes with ice-cold PBS, surface-bound radioactivity was recovered by a 5-min treatment with 0.75 ml of ice-cold 0.2 N acetic acid and 0.15 M NaCl and a subsequent 0.75-ml rinse with the same solution, which was added to the acid fraction. Internalized radioactivity was determined by subsequent solubilization of the cells in 1.5 ml of 0.2 N NaOH and 0.1% SDS. Both the acid and the detergent fractions were analyzed in a gamma-radiation counter. Background binding was determined in the presence of a 100-fold excess of unlabeled EGF.

Receptor internalization. Cells were grown for 2 days to subconfluency in 2.2-cm-diameter wells and were incubated with 100 ng of EGF per ml for various times in serum-free binding medium containing 0.5% BSA and 50 mM HEPES (pH 7.3). Cells were washed twice in binding medium at 0°C and incubated in 0.5 ml of the same medium at 4°C for 16 h in the presence of 0.25 pM 125 I-EGF at 50 TBq/mmol. Cells were washed three times with ice-cold PBS solubilized in 1 ml 0.2 N NaOH and 0.1% SDS, and the radioactivity was determined in a gamma-radiation counter.

Receptor degradation. Subconfluent cells were metabolically labeled with [35 S]methionine for 20 h, rinsed, transferred into normal culture medium, and incubated with EGF (0.5 μ g/ml) for various times. Receptors were immunoprecipitated with MAb108 and analyzed on SDS-7% polyacrylamide gels. The amount of receptor protein was quantified by scintillation counting.

[3 H]thymidine incorporation. Confluent cell monolayers in 12-well (diameter, 2.2 cm) culture dishes were grown to quiescence in 1 ml of medium containing 0.5% fetal bovine serum for 24 h. DNA synthesis was stimulated by adding various amounts of mouse EGF and was measured 18 h later by a 4-h pulse-labeling with 0.5 μ Ci of [methyl- 3 H]thymidine at 3 TBq/mmol. Cells were washed three times with ice-cold PBS, and soluble radioactivity was extracted with 1 ml of 10% trichloroacetic acid for 1 h at 4°C followed by one wash with the same solution at 4°C. Cells were solubilized in 0.5 ml of 0.2 N NaOH and 0.1% SDS for 1 h at 37°C, and the lysate was brought to neutral pH with Tris buffer. The incorporated radioactivity was determined in a liquid scintillation counter with 10 ml of aqueous scintillation cocktail.

Cell growth in serum-free medium. Stable expressing cells or control-transfected cells (30,000 per dish) were plated in

FIG. 1. Predicted structure of normal and chimeric expression products. Proteins are shown spanning the plasma membrane aligned at their putative transmembrane domains, as predicted from their primary structure. Single or clustered cysteine residues are represented by open circles and open boxes, respectively, and the tyrosine kinase domains are indicated by shaded boxes. The attachment of the pp60^{v-src} protein to the cell membrane is represented by a zigzag line, and the amino acids at the junction of the two chimeras are indicated.

35-mm-diameter culture dishes and transferred after 24 h to serum-free DMEM in the presence of either transferrin (10 µg/ml; Sigma), transferrin and EGF (10 ng/ml; Collaborative Research), transferrin and insulin (1 µg/ml; Eli Lilly), or transferrin, insulin and EGF. Cell numbers were determined after 5 days with a cell counter.

Cell focus formation. NIH 3T3 cells (2×10^5 per dish) were plated into 35-mm-diameter dishes, grown for 1 day, and placed into fresh medium. After 4 h, the subconfluent monolayers were transfected by calcium phosphate coprecipitation (19) of 5 µg of high-molecular-weight NIH 3T3 carrier DNA and of 100 ng of receptor construct or v-fms control expression plasmid in the presence or absence of 100 ng of transforming growth factor α (TGF- α) expression plasmid (10). After 24 h, cells of each 35-mm-diameter dish were transferred to three 60-mm-diameter dishes and subsequently cultured in DMEM with 5% fetal calf serum. The medium was changed every 3 to 4 days, and foci were scored after 16 days. Cells were stained with crystal violet after 3 weeks.

Anchorage-independent cell growth. Subconfluent stably transfected cells were trypsinized and plated (10^5 cells per 6-cm-diameter dish) in the presence or absence of 10^{-8} M EGF in 10 ml of MEM. Each dish contained 15% fetal bovine serum and 0.25% agar on a bottom layer of 0.5% agar in 5 ml of MEM. Colonies of 20 or more cells were scored after 20 days.

RESULTS

cDNA fusion and transfection. Cloned HER cDNA (52) and v-fms (20) or v-src (7) coding DNA sequences were used in combination with simian virus 40 early transcriptional elements to generate expression plasmids coding for the complete HER and the chimeras EFR and ESR (Fig. 1). EFR consists of most of the extracellular domain of HER (amino acids [aa] 24 to 590 excluding 32 aa residues that precede its transmem-

FIG. 2. [³⁵S]Met metabolically labeled expression products on SDS-7% polyacrylamide gels. HER, EFR, or ESR proteins have been immunoprecipitated from detergent lysates of two independently transfected cell lines (a and b) and control cells (3T3) by using the EGF receptor mouse monoclonal antibody MAb108. Size markers (in kilodaltons) are indicated.

brane domain) joined with the transmembrane and cytoplasmic domains of gp140^{v-fms} including a 31-aa section of its extracellular domain (aa 973 to 1434) which contains cysteine 975. ESR consists of the extracellular and transmembrane domains of HER plus 44 aa of the HER cytoplasmic domain (aa 24 to 688), while the HER kinase domain had been replaced by the tyrosine kinase domain of pp60^{v-src} (aa 264 to 530) at a similar distance from the HER transmembrane domain. The expression plasmids which contain independent expression units for neomycin resistance and DHFR (6, 49) were introduced into mouse NIH 3T3 fibroblasts by calcium phosphate precipitation, and stable transfectants were first selected with G418. In order to increase receptor expression levels, cDNA copy numbers per cell were subsequently amplified with increasing doses of methotrexate, which selects for cells expressing multiple copies of the DHFR cDNA. Three independently isolated cell lines were analyzed for each construct, and representative data for one or two cell lines (a and b) are presented in each figure (see Table 1).

Expression, cell surface routing, and ligand binding. The cDNA constructs were first tested for faithful expression, and their products were assayed for proper transport to the cell surface. Stable expressing cells were metabolically labeled with [³⁵S]methionine, and proteins were immunoprecipitated with HER-specific monoclonal antibodies, separated on SDS polyacrylamide gels, and analyzed by autoradiography. Specific bands were detected for all constructs with mobilities as expected for the chimeras and for normal HER (Fig. 2). The proteins migrated at positions consistent with glycosylated expression products of 170 kDa for HER, 160 kDa for EFR and 110 kDa for ESR. A second minor band of 150 kDa was detected in cells expressing HER and EFR, which may represent HER and EFR processing precursor forms which have not been investigated.

Transfection of NIH 3T3 cells led, for each of the constructs, to increased numbers of cell surface EGF-binding sites above the endogenous mouse fibroblast background, demonstrating that the constructs directed biosynthesis of normal or chimeric EGF receptors, which were transported to the cell surface in the correct transmembrane orientation. Scatchard analysis of [¹²⁵I]-EGF binding to HER-expressing cells revealed a curvilinear plot with two characteristic binding affinities (K_d s) of 0.005 and 1.2 nM (Fig. 3A; Table 1), while only one form of binding site with a K_d of 4 nM for EFR and with a K_d of 2 nM for ESR (Fig. 3B and C; Table 1) was detected. Thus, combination of the HER extracellular domain with gp140^{v-fms} and pp60^{v-src} protein sequences has a pronounced effect on its ligand-binding characteristics.

FIG. 3. (A to C) Scatchard analysis of ligand binding to HER (A), EFR (B), and ESR (C) before and after treatment with TPA. Subconfluent cells were incubated in the presence (interrupted line, open symbols) or absence (continuous line, black symbols) of 100 nM TPA, and 125 I-EGF binding was subsequently determined. A computer program by S. Felder (Rorer Biotechnology, Inc.) was used for Scatchard analysis. Two independently transfected cell lines for each construct were analyzed with consistent results, and the data for one cell line are presented. Double measurements were made for each datum point at 5% error, and background binding was determined at a 100- to 1,000-fold excess of unlabeled ligand. (D) Time dependence of TPA-modulated ligand binding to HER, EFR, and ESR. Subconfluent cells were incubated for the times indicated with 100 nM TPA before 125 I-EGF binding was measured at 4°C. Background binding was determined in the presence of a 1,000-fold excess of unlabeled EGF, and double measurements were obtained for each datum point at 5% variation. Two independently transfected cell lines were analyzed with consistent results, and the data for cell lines HER b (■), EFR a (◇), and ESR b (▼) are presented.

To investigate whether gp140^{v-fms} and pp60^{v-src} cytoplasmic structures are able to functionally interact with HER extracellular sequences, the effect of cell exposure to the phorbol ester TPA was examined. PKC, which is activated by TPA, has been shown to phosphorylate the cytoplasmic domain of HER at threonine and serine residues (3, 9, 24, 26, 63). For wild-type HER, this phosphorylation causes the loss of the high-affinity EGF-binding state (27, 33, 48). Scatchard analysis of 125 I-EGF binding to HER after TPA induction for 1 h confirmed the loss of high-affinity sites with constant cell surface receptor numbers (Fig. 3A; Table 1). For EFR, the EGF-binding affinity remained almost unaffected, whereas the number of receptors at the cell surface was reduced to about 40% upon TPA stimulation (Fig. 3B; Table 1). For ESR, which still contains the PKC target residue Thr-654 of HER, surface receptor numbers and EGF binding affinity remained unchanged after TPA treatment (Fig. 3C; Table 1).

Both the loss of high-affinity EGF-binding sites of HER and the reduction of EFR cell surface receptor numbers were fully established as early as 5 min after exposure to TPA and remained constant thereafter (Fig. 3D). Since the normal cellular protein gp165^{c-fms}, but not the transforming protein gp140^{v-fms}, is similarly internalized in response to TPA (60), it appears that the extracellular domain of HER restores the TPA-responsive downmodulation of gp140^{v-fms}. This finding demonstrates that heterologous gp140^{v-fms} and HER domains can interact across the plasma membrane and suggests that the mode of receptor downmodulation is predefined by the cytoplasmic domain but requires activation by extracellular sequences.

Ligand activation of tyrosine kinase. Functional cell surface receptors of the tyrosine kinase family translate extracellular ligand interaction into intracellular activation of kinase functions. This is thought to result first in autophosphorylation and

TABLE 1. Receptor expression levels and binding characteristics^a

Cell line	Receptors per cell (10 ³) for the indicated affinity state ^b			<i>K_d</i> (nM)		
	-TPA		+TPA	-TPA		+TPA
	High	Low	Low	High	Low	Low
HER a	2.7	820	780	0.005	1.3	1.8
HER b	6.1	560	540	0.005	1.2	1.6
EFR a		102	37		4.8	6.1
EFR b		74	29		3.5	4.7
ESR a		1,333	1,467		1.9	1.7
ESR b		229	241		1.8	2.1

^a Values for EGF receptors (HER) and chimeric receptors (EFR and ESR) were determined by cell surface binding of iodinated EGF in the presence (+TPA) or absence (-TPA) of TPA followed by Scatchard analysis using a computer program developed by S. Felder (Rorer Biotechnology, Inc.). Two affinity classes of binding sites (high, low) were detected only in cell lines HER a and HER b in the absence of TPA. High-affinity EGF-binding sites were abolished in the presence of TPA. The values for receptor numbers and dissociation constants (*K_d*) varied up to 20% in parallel determinations.

^b The receptor numbers presented include endogenous mouse EGF receptors, 1.6×10^4 in the NIH 3T3 line used in these experiments. This number was found to be constant in the transfected cell lines analyzed.

subsequently in phosphorylation of cellular substrate proteins. To test whether the EFR and ESR chimeras were functional in transmitting external signals across the cell membrane into the cell, protein kinase activities were measured in vitro and in intact cells. In vitro phosphorylation was tested by adding EGF to detergent lysates of expressing cells, immunoprecipitating the chimeric proteins, and incubating the precipitates in the presence of [γ -³²P]ATP. Analysis of these samples by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that HER and EFR displayed basal autophosphorylation activities that were stimulated three- and twofold, respectively, by EGF (Fig. 4A), while ESR autophosphorylation activity was not detectable. However, ESR appeared to phosphorylate Src antibodies (most likely their heavy chains) directed against the pp60^{v-src} tyrosine kinase domain, as previously observed with pp60^{v-src} (31). This antibody phosphorylation, however, was found not to be EGF responsive (Fig. 4A).

Protein phosphorylation was also determined under semi-in vivo conditions after partial permeabilization of cell membranes with the detergent digitonin in the presence of EGF and [γ -³²P]ATP. Phosphorylated proteins were immunoprecipitated with EGF receptor-specific or phosphotyrosine antibodies and analyzed by SDS-PAGE (Fig. 4B and C). All receptor constructs were efficiently phosphorylated under these conditions and were readily recognized by HER-specific antibodies (Fig. 4B). Phosphotyrosine-specific antibodies, however, failed to precipitate ESR (Fig. 4C). Analysis with both antibodies indicated catalytic stimulation of HER and EFR by EGF added to digitonin-treated cells, while ESR either was not detected (Fig. 4C) or exhibited only basal, EGF-unresponsive levels of ³²P incorporation (Fig. 4B). Like pp60^{v-src}, ESR may be predominantly phosphorylated on Ser (25), which in combination with the limitations of the phosphotyrosine-specific antibodies used may explain the lack of signal in Fig. 4C.

In addition to the receptor constructs, phosphotyrosine-specific antibodies detected a variety of cellular phosphoproteins with molecular weights (MWs) between 64,000 and 20,000, with increased intensities after EGF stimulation (Fig. 4C). Three of these, at MWs of 55,000, 35,000, and 28,000,

FIG. 4. (A) In vitro phosphorylation of receptor constructs. Receptors in detergent cell lysates were incubated with (+) or without (-) EGF and were immunoprecipitated with monoclonal EGF receptor antibody MAb108 from cell line a or b or control cells (3T3). Precipitates were incubated with [γ -³²P]ATP at 0.15 μ M for HER and ESR or 50 μ M for EFR. The ESR kinase was also tested in the presence of specific rabbit polyclonal antibodies against p60^{v-src} (src, ab). Size markers (in kilodaltons) are indicated. (B and C) Ligand stimulation of receptor tyrosine kinases (B) and cellular phosphoproteins (C) in digitonin-permeabilized cells. Cells were incubated with [γ -³²P]ATP in the presence (+) or absence (-) of EGF while membranes were partially permeabilized with digitonin. (B) Receptors HER, EFR, and ESR were immunoprecipitated with EGF receptor monoclonal antibody MAb108 and were analyzed on an SDS-7% polyacrylamide gel. (C) Phosphotyrosine-containing cellular proteins were immunoprecipitated with phosphotyrosine monoclonal antibody MAb5E2 and analyzed on an SDS-7 to 15% polyacrylamide step gradient gel. Size markers (in kilodaltons) are indicated.

might be substrates shared by HER and EFR, whereas polypeptides with MWs of 64,000, 40,000, 30,000, and 20,000 specifically appeared in conjunction with HER expression, and a band with an MW of 48,000 was detected only after EFR expression. It is unlikely that most of these bands represent direct substrates of HER or EFR. They do not match the phosphoprotein pattern that has been observed in response to CSF-1 (46), and no attempt has been made to identify any of the underlying proteins. Only one shared direct protein substrate, the p85 subunit of phosphatidylinositol 3'-kinase, which is, however, not phosphorylated by the receptor, has been described to date for HER and gp165^{c-fms} (56). The observed phosphoprotein pattern simply suggests that like HER, EFR is a ligand-responsive receptor, which undergoes autophosphorylation, stimulates phosphorylation of cellular proteins on tyrosine, and may share some biological activities with the parental EGF receptor such as a mitogenic signal, while other biological activities appear to be distinct and may be involved in HER- or Fms-specific functions. These experiments indicate that the heterologous domains involved in ligand binding and signal generation are able to form a functional EFR chimera and suggest that HER and gp140^{v-fms} employ compatible mechanisms of transmembrane signaling.

Ligand internalization, receptor downregulation, and stability. EGF internalization by receptor constructs was measured by exposing cells to a saturating amount of ¹²⁵I-EGF at 37°C. At various times, cell surface-associated radioactivity was determined by an acid wash and compared with the amount of internalized ligand in solubilized cells. All receptor constructs internalized EGF; however, the efficiency of ligand internalization was reduced in cell lines with high receptor expression levels, suggesting that the endocytotic machinery had become saturated (Fig. 5A). Half-maximal internalization was observed in most cell lines after about 5 min. This time was increased to about 10 min in those cell lines (HER a and ESR a) that expressed particularly high receptor levels (about 10⁶ per cell) (Fig. 5A; Table 1). EGF internalization reached a maximum level of about 60% in EFR-expressing cells after 15 to 30 min and slowly declined thereafter, presumably because of accelerated EFR degradation (Fig. 5C, E, and F), whereas in HER- and ESR-expressing cells, saturation was reached after 1 to 2 h (Fig. 5A).

The time course for total, cell surface-associated and internalized ligand in HER-expressing cell lines showed that the disappearance of cell surface-bound ligand (about 40% after 1 h) was reciprocal to the increase of internalized ligand (about 60% after 1 h) and resulted in constant amounts of total (surface-bound plus internal) ligand (Fig. 5B). A very similar result was obtained for EFR-expressing cell lines except that the numbers for total and surface-bound ligand were found to decrease after 30 min (Fig. 5C). Presumably, this is caused by accelerated downregulation and degradation of the EFR protein (Fig. 5E and F). In contrast, ESR-expressing cells displayed constant amounts of cell surface-bound ligand, while the internalized ligand and consequently the total amount of surface-bound and internal ligand combined increased in time (Fig. 5D to F).

In parallel, the ability of EGF to induce the disappearance of chimeric receptors from the cell surface was measured by triggering internalization with a saturating amount of nonradioactive EGF at 37°C. After various times, cells were cooled to 4°C, and the level of receptors present at the cell surface was determined by binding of ¹²⁵I-EGF. The receptor levels at the surface of cell lines HER a and EFR a decreased to 60% and 40%, respectively, of their original levels after 1 h of EGF treatment (Fig. 5E), consistent with the observed ligand inter-

nalization presented above (Fig. 5A to C). In contrast, EGF binding to the expressing cell line ESR b was not significantly decreased in time (Fig. 5E). Probably, ESR is efficiently recycled, thereby mediating efficient EGF internalization while average numbers of cell surface binding sites hardly change (Fig. 5D).

The EGF receptor is downregulated in response to EGF and is degraded at an increased rate upon EGF-stimulated receptor internalization in a variety of cell types (8, 13, 50). To test whether EGF binding targeted chimeric receptors into a degradative pathway or simply slowed their retrieval from endocytic compartments, cells were metabolically labeled for 20 h with [³⁵S]methionine and subsequently incubated with unlabeled methionine for various times in the presence or absence of EGF. Receptors were immunoprecipitated from detergent cell lysates with HER-specific antibodies and separated on SDS polyacrylamide gels, and receptor bands were isolated and quantitatively evaluated by scintillation spectroscopy. In stably expressing NIH 3T3 cell lines, the half-life of HER was 14 h, which decreased to 3 h after EGF stimulation (Fig. 5F). The presence of gp140^{v-fms} sequences in EFR altered these times to 5 and 2 h, respectively. In contrast, the presence of the ligand-nonresponsive pp60^{v-src} kinase in ESR led to the same half-life (about 15 h) as that observed for normal HER, which remained unresponsive to EGF. Presumably, a ligand-activated tyrosine kinase is necessary to decrease the receptor half-life. These results are reminiscent of studies which demonstrated that a tyrosine kinase-deficient, mutated HER mediates ligand internalization but escapes transport to lysosomes and subsequent degradation and is quickly recycled to the cell surface (21, 22).

Mitogenic response and growth factor requirement. The physiological activity of the receptor constructs was tested by measuring the mitogenic response of transfected NIH 3T3 cells to EGF. Confluent cell monolayers grown to quiescence in 0.5% serum were stimulated with EGF for 18 h followed by labeling with [³H]thymidine for 4 h to measure EGF-stimulated DNA synthesis. A clear response due to the background level of endogenous mouse EGF receptors (Fig. 6A) was detected in control NIH 3T3 cells. A threefold-higher, ligand-dependent response which was half-maximal at 0.1 nM EGF was observed in HER-expressing cells. EFR-expressing cells could not be starved to quiescence and always displayed strong mitogenic activity but responded only marginally to EGF stimulation. In contrast, expression of ESR inhibited the endogenous mitogenic response to EGF, suggesting that the ligand-unresponsive ESR interfered with the cellular mitogenic machinery. Such interference might have been caused by formation of signaling-incompetent mouse EGF receptor-ESR heterodimers.

The constitutive mitogenic activity observed in EFR-expressing cells (Fig. 6A) suggested that these cells may display reduced growth factor dependence. This was tested directly in serum-free cell culture in the presence of defined growth factors. Cells (3 × 10⁴) were incubated in the presence of combinations of transferrin, EGF, and insulin, and cell numbers were determined after 5 days. As shown in Fig. 6B, all cell lines displayed a maximal rate of cell doubling when supplemented with all three factors. Numbers of control cells, cells expressing HER, and cells expressing ESR increased three-, six-, and twofold, respectively, but these cells did not divide in the presence of transferrin alone. Maximal growth of ESR-expressing cells was somewhat reduced when compared with control cells, consistent with the decreased [³H]thymidine incorporation observed with this construct. EFR expression led to a threefold increase in cell numbers when supplemented

FIG. 5. (A) EGF-induced ligand internalization in HER a (□), HER b (■), EFR a (◇), EFR b (◆), ESR a (▽), and ESR b (▼) cell lines. Subconfluent cells were incubated with 2 nM ^{125}I -EGF for the times indicated, and surface-bound radioactivity was measured in the supernatant of an acid wash before the internalized radioactivity was determined by cell solubilization. Background binding was assayed in the presence of a 100-fold excess of unlabeled EGF. The average of double measurements, which varied by 10%, is presented for each datum point. The total of surface-bound and internalized radioactivity is defined as 100% for each cell line. (B to D) Time course of surface-bound, internal, and total (surface bound plus internal) ligand in HER b (B), EFR a (C), and ESR b (D) cell lines. Ligand internalization has been measured as described in the legend to panel A, but ^{125}I -EGF binding data were analyzed to obtain graphs for total (○), surface-associated (▽), and internal (△) radioactivity. Consistent results were obtained for the second independent cell line of each construct (not shown). (E) EGF-induced receptor internalization in HER b (■), EFR a (◇), and ESR b (▼) cell lines. Subconfluent cells were incubated with 16 nM EGF for

FIG. 6. (A) Mitogenic EGF response of expressing cell lines HER a (□), HER b (■), EFR a (◇), EFR b (◆), ESR a (▽), and ESR b (▼) and of control cells (○). Confluent cells were grown to quiescence in culture medium with 0.5% fetal calf serum. Stimulation of DNA synthesis by the addition of various amounts of EGF was monitored 18 h later by a 4-h pulse-labeling with [*methyl*-³H]thymidine and subsequent determination of trichloroacetic acid-precipitable radioactivity. The average of double measurements, which varied by 10%, is shown for each datum point. (B) Factor requirement for growth in serum-free medium. Thirty thousand cells of each line were plated and subsequently grown in serum-free culture medium supplemented with transferrin (open bars), transferrin and EGF (dotted bars), transferrin and insulin (hatched bars), or transferrin, EGF, and insulin (closed bars). Cell numbers were determined in a cell counter after 5 days and varied by 10% in double measurements for each datum point.

with transferrin alone, which were only slightly increased further in the presence of all three growth factors (Fig. 6B), suggesting that the mitogenic activity shown in the previous experiment correlates with a minimal growth factor requirement of EFR-expressing cells.

Cell morphology, anchorage-independent growth, and focus formation. To test whether EFR-expressing cells appeared transformed by other criteria and to investigate the transforming potential of ESR, the cell morphology, formation of foci, and anchorage dependence of cell growth were analyzed. In comparison with control cells in normal culture, significant changes in cell morphology were not detected except for EFR-expressing cells, which displayed a more spindle-shaped phenotype and reduced contact inhibition (not shown). To formally test for the transforming potential of the chimeras, NIH 3T3 cells were transfected with expression plasmids and the formation of cell foci was scored after 16 days. Foci were readily induced by EFR (around 300/μg of DNA) and most prominently by gp140^{v-fms} (around 2,000/μg of DNA) but were not observed in control-transfected or HER-transfected cells (Fig. 7A; Table 2). Cotransfection of receptor expression constructs with a TGF-α expression plasmid in a parallel experiment, however, resulted in the formation of foci in HER-transfected cells (740/μg of DNA) while it did not significantly change the transforming potential of EFR and gp140^{v-fms}. No foci were observed in control or ESR-transfected cells (Fig. 7A; Table 2). These results confirm the ligand-induced transforming potential of HER (11, 38, 57) and demonstrate a high basal transforming activity of EFR reminiscent of the characteristics of the parental gp140^{v-fms} (39).

To test for anchorage-independent cell growth as an additional criterion for the transformed phenotype, expressing cell lines were plated in soft agar (0.25%) in the presence or absence of 10 nM EGF in medium containing 15% fetal bovine serum and colonies were scored after 20 days. In this assay, small but increasing numbers of colonies were induced only by EFR with increasing expression levels (Fig. 7B; Table 2) compatible with the basal transforming activity of EFR described above (39). In the presence of added EGF, colonies were formed in small numbers even in control cells and in small but greater numbers with increasing expression levels of ESR. This may suggest that the endogenous mouse EGF receptor and the particularly high cellular levels of ESR (ESR b, 2.3×10^5 per cell; ESR a, 1.3×10^6 per cell) result in some transforming activity when the cells are stimulated with high doses of EGF. In contrast, large numbers of colonies were induced by HER and EFR in the presence of high EGF concentrations, elevated up to 1,000-fold over control numbers. Colony numbers always increased with increasing expression levels. This confirms the ligand-dependent transforming potential of HER and indicates that while EFR displays a strong mitogenic signal already in the absence of ligand like gp140^{v-fms}, this activity is still ligand responsive with respect to anchorage-independent cell growth, like the catalytic activity.

the times indicated, and surface-bound radioactivity was determined with ¹²⁵I-EGF at 4°C. Background EGF binding was assayed in the presence of a 100-fold excess of unlabeled EGF. The average of double measurements, which varied by 10%, is presented for each datum point. Surface binding at 0 min is defined as 100% for each cell line. (F) Ligand-stimulated receptor degradation. Subconfluent cells were metabolically pulse-labeled for 20 h with [³⁵S]methionine, and receptor degradation was measured by a chase in normal culture medium in the presence (underlined time points) or absence of EGF for the times indicated in hours. Receptors were immunoprecipitated with a specific monoclonal antibody, MA108, directed against the extracellular domain of HER and were analyzed on an SDS-7% polyacrylamide gel. Receptor degradation was studied with the same result in two independently transfected cell lines, data for one of which are presented. Receptor numbers have been quantified by scintillation counting in order to determine the half-life of the receptor constructs (see the text). Size markers (in kilodaltons) are indicated.

FIG. 7. (A) NIH 3T3 cell focus formation. Subconfluent NIH 3T3 monolayers in 35-mm dishes were transfected with HER, EFR, ESR, v-fms, or control expression plasmids (—). In parallel, cells were cotransfected with a TGF- α expression plasmid. Cells were stained with crystal violet after 3 weeks. (B) Ligand-stimulated anchorage-independent cell growth. Cell lines HER a and b, EFR a and b, and ESR a and b and control cells (NIH 3T3) were trypsinized and plated in the presence (EGF) or absence (—) of EGF in MEM containing 15% fetal calf serum and 0.25% agar on a 0.5% agar bottom layer. Colonies were scored after 20 days. The width of the panels corresponds to 3.3 mm.

DISCUSSION

To investigate the functional compatibility and the specific roles of distinct receptor tyrosine kinase domains, two chimeric EGF receptors have been designed by in vitro cDNA combination. EFR is composed of most of the EGF receptor extracellular ligand-binding domain fused to a short extracellular sequence and the transmembrane and cytoplasmic domains of the FeSV transforming protein gp140^{v-fms}, the oncogenic counterpart of CSF-1 receptor, gp165^{c-fms} (5, 47). ESR consists of extracellular, transmembrane, and juxtamembrane

EGF receptor sequences fused to the tyrosine kinase domain of pp60^{v-src}, the transforming protein of Rous sarcoma virus (23), which does not span the plasma membrane and is membrane associated via a myristylated domain (34). Both chimeras and normal EGF receptors were stably expressed in NIH 3T3 fibroblasts to study the interaction and functional consequences of the heterologous cytoplasmic and extracellular domains within a membrane-spanning cell surface receptor.

The results demonstrate that both EFR and ESR chimeras, like normal HER, are faithfully synthesized and subsequently

TABLE 2. Anchorage-independent cell growth and focus formation

Construct	Foci per μ g of DNA ^a		Cell line	Soft agar colonies per 100,000 cells ^b	
	– Ligand	+Ligand		– Ligand	+Ligand
HER	<5	740	a	1	1,120
			b	2	530
EFR	333	250	a	86	4,800
			b	13	1,980
ESR	<5	<10	a	2	93
			b	0	40
Control	<5	<10		0	11
v- <i>fms</i>	2,500	1,600		ND ^c	

^a Numbers of cell foci were determined after transfection with HER, EFR, ESR, v-*fms*, or control expression plasmids (–ligand) and after cotransfection with a TGF- α expression construct (+ligand). Foci were scored after 16 days in triple determinations at 20% variation, and average numbers per microgram of DNA used in the transfection are presented.

^b Soft agar colonies were counted 20 days after the plating of 100,000 cells in medium containing 0.25% agar in the presence (+ligand) or absence (–ligand) of EGF. Colonies of 20 or more cells were scored in triple determinations at 20% variation, and average numbers are presented.

^c ND, not determined.

processed and transported to the cell surface where they expose EGF-specific ligand-binding competent structures into the extracellular space. The ligand-binding properties were found to be altered in both chimeras compared with the normal human EGF receptor, as a consequence of the heterologous kinase domain. Normal EGF receptors display high- and low-affinity EGF-binding sites at the cell surface (27, 48). The high-affinity sites are downmodulated upon PKC-mediated receptor phosphorylation at threonine and serine residues, in particular at Thr-654 (3, 9, 24, 26, 63). Replacement of the HER kinase domain with the pp60^{v-src} kinase resulted in the formation of a single low EGF-binding affinity for ESR which was unaffected by TPA treatment. It is unlikely that the pp60^{v-src} domain in ESR is phosphorylated by PKC since it does not contain the PKC target amino acid Ser-12 (18). The presence of the juxtamembrane domain of HER including Thr-654 is not sufficient to allow modulation of the EGF-binding affinity by PKC in a kinase-deficient HER mutant which in contrast to ESR displays high- and low-affinity EGF binding sites and is not phosphorylated by PKC (33). Constitutive phosphorylation of Thr-654 in ESR could be one reason for the loss of high-affinity EGF binding sites, but this has not been investigated. This study indicates that the presence of the (EGF-unresponsive) pp60^{v-src} kinase domain abolishes high-affinity EGF binding sites in a dominant fashion, similarly to the EGF-responsive insulin receptor kinase domain in an EGF-insulin receptor chimera (36), while a kinase-deficient HER mutant displays the normal high and low EGF-binding affinities (21).

Transmembrane and cytoplasmic sequences of gp140^{v-fms} in EFR similarly affected EGF binding in a dominant fashion and resulted in the formation of a single low EGF-binding affinity comparable with the characteristics of ESR. TPA treatment caused a 2.5-fold reduction in numbers of EFR cell surface EGF binding sites without affecting the binding affinity. This is consistent with the reported downmodulation of the CSF-1 receptor, gp165^{c-fms} (45), and in contrast to the lack of downmodulation of gp140^{v-fms} in response to CSF-1 or TPA treatment (60). Replacement of the amino terminus of gp140^{v-fms} by 308 aa residues of gp165^{c-fms} was shown to restore ligand- but not TPA-induced downmodulation of the resulting c-*fms*/v-*fms* chimera (39). The opposite response was observed for a

kinase-defective mutant of gp165^{c-fms} (Lys-616 replaced by Met) which was downmodulated by TPA but not by CSF-1. Intermediates of receptor degradation were detected upon TPA stimulation but not upon CSF-1 treatment (12). These data demonstrate independent mechanisms for ligand-induced and TPA-induced downmodulation of gp165^{c-fms} (12). Conversion of tyrosine 969 to phenylalanine in gp165^{c-fms} did not affect receptor downmodulation, although it potentiated the transforming efficiency in cells cotransfected with CSF-1 (35).

Downmodulation and degradation in response to CD2 T-cell antigen antibodies has been reported for a chimera composed of an extracellular CD2 domain fused to gp165^{c-fms} transmembrane and cytoplasmic sequences (43). In contrast, chimeras composed of the extracellular domain of the major erythrocyte membrane sialoglycoprotein glycophorin A, cytoplasmic sequences of gp165^{c-fms}, and transmembrane sequences of either origin were internalized but not degraded in response to glycophorin A antibodies, suggesting that extracellular sequences play some role in the intracellular fate of gp165^{c-fms} (29). Mutations in its kinase domain showed that CSF-1-induced internalization and degradation of gp165^{c-fms} are separable events (2). EFR in this study was efficiently internalized and degraded in response to EGF, as was the normal gp165^{c-fms} in response to CSF-1, suggesting that the 590 extracellular amino-terminal aa residues of HER fully restore ligand- and TPA-induced downmodulation of gp140^{v-fms}, as in gp165^{c-fms}, with respect to both mechanisms. Consequently, the cytoplasmic domain of gp140^{v-fms} has retained the potential to interact with PKC to undergo TPA-mediated downmodulation in cooperation with appropriate extracellular sequences. The EGF receptor extracellular domain provides the required functional interactions in EFR.

In contrast, replacement of the HER kinase domain by the pp60^{v-src} tyrosine kinase in ESR leads to lack of biological activity and severely alters cellular routing. The finding in this study that ESR bound and internalized EGF while average quantities of cell surface EGF-binding sites and total cellular ESR protein, as judged by immunoprecipitation, remained unchanged suggests that ESR is internalized but escapes degradation in lysosomes and is recycled to the cell surface. In contrast to normal HER, which is internalized, targeted to lysosomes, and degraded in response to EGF, a routing similar to that for ESR has been reported for a tyrosine kinase-deficient HER mutant (15, 21) while a second, distinct kinase-deficient HER mutant failed to undergo ligand-induced internalization (17). EGF receptor internalization is not required for an EGF-induced mitogenic response or for cell transformation, as shown by an internalization-defective mutant (59). These somewhat conflicting reports and the results of this study are still compatible with the idea that a ligand-activated tyrosine kinase may be required for EGF-induced EGF receptor internalization and targeting into the degradative pathway, as in the case of EFR, which may not be replaced by a ligand-unresponsive kinase domain with low activity, as shown for ESR, or by an inactive kinase (15, 17, 21). In the present study, ESR kinase activity was low, was only detected in vitro towards associated pp60^{v-src} antibodies, and was found not to be ligand responsive. The cytoplasmic domain, including the tyrosine kinase, controls EGF receptor intracellular sorting at the multivesicular body (15), and consequently a heterologous cytoplasmic domain plays a critical role in determining the intracellular routing of the receptor in response to the ligand. Depending on its nature, it can protect the EGF receptor from the degradative pathway as shown for an EGF receptor chimera with a cytoplasmic insulin receptor domain (36). The combination of the EGF receptor ligand-binding domain with

the gp140^{v-fms} kinase appears to restore ligand- and TPA-responsive receptor downmodulation as described for normal gp165^{c-fms}, which shares some characteristics of normal EGF receptor intracellular routing (2).

None of the activities of ESR was found to be significantly EGF responsive, suggesting either that the HER and pp60^{v-src} domains used are incompatible or insufficient to form a functional chimeric receptor or that the design of the construct did not allow the formation of a functional protein, since proteolytic fragments containing the pp60^{v-src} tyrosine kinase domain are still catalytically active (30). The undetectable level of autophosphorylation observed for ESR is consistent with early studies of pp60^{v-src}, which could only demonstrate antibody substrate phosphorylation in immunoprecipitates (31). Similarly, ESR phosphorylated pp60^{v-src} antibodies in vitro in this study. Apparently, the substrate-phosphorylating activity of ESR is more pronounced than any residual basal autophosphorylating activity (61), although in vitro autophosphorylation has also been demonstrated for pp60^{v-src} and the observed reaction may depend on the stoichiometry between the kinase and the substrate (32). In vivo phosphorylation of pp60^{v-src} has been shown, however, mainly on Ser residues and to a lesser extent on Tyr residues (25). This could explain why ESR was efficiently precipitated by HER-specific antibodies but not by a phosphotyrosine antibody from digitonin-permeabilized cells. The lack of a ligand response in EFR may have been caused by the absence of the carboxyl-terminal regulatory domain of pp60^{c-src}, which is involved in the regulation of pp60^{c-src} activity but is missing in pp60^{v-src} and may render this transforming protein irreversibly active. Alternatively, parts of the amino-terminal regulatory domain of pp60^{v-src}, which is involved in membrane attachment by myristylation and contains an SH2 and an SH3 domain, may be required for the regulation of the tyrosine kinase activity even in the context of the ESR chimera, since they may not be replaceable by HER sequences (1).

gp165^{c-fms} is a fully ligand-responsive tyrosine kinase, whereas gp140^{v-fms} displays constitutive tyrosine kinase and biological activities that are oncogenic and independent of CSF-1 (44, 60). The major structural difference between these two proteins, the carboxyl-terminal truncation of gp140^{v-fms}, was shown not to be sufficient to fully explain its cell-transforming potential; instead, alterations in its extracellular domain contribute to the constitutive transforming phenotype (39, 41, 62). Point mutations in the extracellular and in the cytoplasmic domains were shown to be required to fully activate the transforming potential of gp140^{v-fms} (40, 62). With the chimera EFR, we have created a molecule that combines features of both gp165^{c-fms} and gp140^{v-fms}. Replacement of the extracellular sequences of gp140^{v-fms} with an EGF binding domain results in a ligand-responsive tyrosine kinase and causes anchorage-independent cell growth upon EGF stimulation. EFR displays additional characteristics of the transformed phenotype already at very low serum EGF concentrations. NIH 3T3 cells expressing EFR are mitogenically stimulated and display elevated [³H]thymidine incorporation into DNA, reduced growth factor dependence, and the formation of cell foci. Most of these characteristics have been shown after expression of gp140^{v-fms} or mutated, oncogenic human gp165^{c-fms} in NIH 3T3 cells, which resulted in cell proliferation and transformation in the absence of any growth factors (42). The mitogenic activity of EFR is comparable to that of chimeras composed of the extracellular domain of gp165^{c-fms} and the cytoplasmic domain of gp140^{v-fms} which show a partially transformed phenotype including cell focus formation with the normal extracellular domain of gp165^{c-fms} while additional activation by point mutations in the regulatory

domain or by the ligand enhances the transformed phenotype and results in anchorage-independent cell growth (39, 40, 62). EFR similarly causes the formation of cell foci at very low serum ligand concentrations, while high EGF concentrations are required for anchorage-independent cell growth.

In contrast, ESR displays a ligand-unresponsive pp60^{v-src} tyrosine kinase which phosphorylates antibody substrates without detectable levels of autophosphorylation. Receptor routing was severely altered, and no significant cell-transforming or other physiological activity has been detected. Even the biological response of endogenous mouse EGF receptors was impaired after ESR expression. This suggests that the EGF-unresponsive ESR interfered with the cellular mitogenic machinery, possibly by the formation of signaling-incompetent mouse EGF-ESR heterodimers, which has not been investigated. Since proteolytic fragments containing the pp60^{v-src} tyrosine kinase domain are still catalytically active (30), the kinase activity of ESR detected in this study is not unexpected. In contrast, the lack of a physiological activity of ESR may be caused by the absence of amino-terminal sequences of pp60^{v-src} which are involved in the regulation and physiological function of pp60^{v-src}, including its modulation by PKC.

The gp140^{v-fms} tyrosine kinase of EFR is EGF responsive in vitro, in digitonin-permeabilized cells, and in vivo. At the same time, the cytoplasmic gp140^{v-fms} domain allows PKC-mediated TPA downmodulation of EFR and alters the EGF-binding characteristics of EFR compared with normal HER by abolishing the high affinity EGF-binding sites. These findings demonstrate that gp140^{v-fms} and HER sequences communicate with each other in both directions and affect the function of the opposed heterologous domain across the plasma membrane. This indicates that while the structures of EGF and CSF-1 receptors differ in both the extracellular and cytoplasmic domains (5, 52), both receptors appear to employ a general and compatible mechanism of transmembrane signaling that can be extended to the transforming protein gp140^{v-fms}. A wealth of data suggests that this mechanism involves ligand-induced, rapid, reversible receptor aggregation and intermolecular allosteric activation which cause a conformational change in the cytoplasmic domain and result in stimulation of the tyrosine kinase (54). In addition, this study confirms that the specificity of the biological signal resides in the cytoplasmic receptor domain, as shown by the distinct effects of HER, EFR, and ESR. This has been shown for a number of receptor tyrosine kinases (64) starting with the EGF and insulin receptors (36, 37).

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