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Chicken Epidermal Growth Factor (EGF) Receptor: cDNA Cloning, Expression in Mouse Cells, and Differential Binding of EGF and Transforming Growth Factor Alpha

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The primary structure of the chicken epidermal growth factor (EGF) receptor was deduced from the sequence of a cDNA clone containing the complete coding sequence and shown to be highly homologous to the human EGF receptor. NIH-3T3 cells devoid of endogeneous EGF receptor were transfected with the appropriate cDNA constructs and shown to express either chicken or human EGF receptors. Like the human EGF receptor, the chicken EGF receptor is a glycoprotein with an apparent molecular weight of 170,000. Murine EGF bound to the chicken receptor with approximately 100-fold lower affinity than to the human receptor molecule. Surprisingly, human transforming growth factor α (TGF-α) bound equally well or even better to the chicken EGF receptor than to the human EGF receptor. Moreover, TGF-α stimulated DNA synthesis 100-fold better than did EGF in NIH 3T3 cells that expressed the chicken EGF receptor. The differential binding and potency of mammalian EGF and TGF-α by the avian EGF receptor contrasts with the similar affinities of the mammalian receptor for the two growth factors.

Control of cell growth is regulated by interaction of soluble growth factors and cell membrane receptors. The study of the specificity of growth factor-receptor interaction and the following steps which lead to the mitogenic responses of the cell is essential for our understanding of normal cell growth control and oncogenesis. Several growth factor receptors possess intrinsic protein tyrosine kinase activity in their cytoplasmic domain, which is activated by ligand binding leading to autophosphorylation, phosphorylation of various cellular proteins, and initiation of pleiotropic responses resulting in DNA synthesis and cell proliferation (reviewed in references 4 and 20 and J. Schlessinger, Biochemistry, in press, and Y. Yarden, and A. Ullrich, Biochemistry, in press). One of the best-characterized growth factor receptors with intrinsic protein tyrosine kinase activity is the epidermal growth factor receptor (EGF-R) (4, 8, 20, 24; Schlessinger, in press). The human EGF-R (HER) recognizes equally well at least three distinct growth factors: epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), and vaccinia virus growth factor (4, 2, 21). Analysis of the cellular functions of the EGF-R was greatly facilitated by the cloning (23) and expression of HER cDNA in animal cells, including murine NIH 3T3 cells (6, 7, 11–13, 16). Various deletions and point mutations in different portions of the receptor allow localization of some of the important functions of the EGF-R (reviewed in reference 20 and Schlessinger, in press).

Another approach to analyzing the structure-function relationships of the receptor molecule is to compare EGF-R of distant evolutionary origin when expressed in the same cellular environment. Modification of biological functions such as ligand recognition due to divergence of primary structure may open up experimental avenues to assignment of functions to specific receptor domains (17). Exchange of cDNA segments between such receptors and construction of chimeric receptors may enable the identification of the ligand-binding domain of the EGF-R. To examine this experimental approach, we have cloned and sequenced the complete cDNA of the chicken EGF-R (CER) and compared it with that of the HER. The CER was constructed in the same vector previously used to express the HER (12, 13), and the construct was expressed in NIH 3T3 cells previously shown to be devoid of endogenous EGF-R (6, 7). Our results demonstrated that, like, the HER, the CER was expressed as a 170-kilodalton glycoprotein in NIH 3T3 cells. However, the CER bound murine EGF with an approximately 50-fold lower affinity than did the HER. Surprisingly, the binding affinity of TGF-α for the CER was equal to, or even greater, than its affinity for the HER. TGF-α also stimulated the kinase activities of the CER and the HER to similar extents. Furthermore, TGF-α was 100-fold more effective than EGF in inducing DNA synthesis in CER-expressing cells. The differential binding and response of the avian EGF-R to the mammalian EGF or TGF-α raises interesting questions regarding the evolutionary divergence of these growth factors. It also provides useful parameters for mapping the ligand-binding regions after domain exchange between the two receptors.

MATERIALS AND METHODS

Mouse EGF was from Toyobo Co., and human TGF-α was either a recombinant TGF-α from Genentech, Inc., or a synthetic TGF-α from Bachem. ^{125}I labeling was by the chloramine-T method to a specific activity of 100,000 to 200,000 cpm/ng. The concentrations of growth factors were determined by amino acid analysis, and their specific activities were determined by displacement titration of cell-bound ^{125}I-labeled growth factor at saturation by unlabeled growth factor.

A cDNA library was prepared by oligo(dT) priming of

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mRNA from chicken (SPASAS) embryo fibroblasts. The double-stranded cDNA was ligated to *Eco*RI linkers and cloned in λgt11 (19). Preparation of phage and plasmid DNA, screening libraries, and probes was by standard methods (14). DNA sequencing was by the chain termination method (18) on M13 subclones of the cDNA, and both strands were sequenced. Preparation of full-length cDNA in the expression vector was performed as follows. The 3.7-kilobase (kb) *Eco*RI cDNA fragment (see Fig. 1) was subcloned into the *Eco*RI site of Bluescript (Stratagene, San Diego, Calif.). The 5′ *Eco*RI site was eliminated by digestion with *Sac*I, which cleaves at the polylinker of the vector and at the 5′ noncoding region (nucleotide 258). The two ends were ligated by a *Sac*I linker which also contains *Xba*I and *Xho*I sites:

CTCTAGACTCGAGACT
TCGAGAGATCTGAGCTC

To this plasmid the 0.7-kb *Eco*RI cDNA fragment (see Fig. 1) was ligated at the unique *Eco*RI site at the 3′ end of the cDNA. The correct orientation was checked by digestion with *Hin*dIII and sequencing. The 4.4-kb cDNA insert was prepared from this plasmid by *Xho*I digestion (one *Xho*I site at the *Sac*I linker and one at the Bluescript polylinkers) and inserted into the unique *Xho*I site of the modified pLSV (12) as previously described. The resultant plasmid, pLSY, was prepared for transfection by the CsCl method. This construct contains 34 base pairs of the 5′ untranslated region and 450 base pairs of the 3′ untranslated region which does not include the polyadenylation site.

Transfections. NIH 3T3 cells (clone 2.2) were grown in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum. Dishes (10-cm diameter) of cells were cotransfected with a mixture (10 μg:1 μg) of pLSY and pSV2Neo DNA per dish by the calcium phosphate precipitation technique (26). Two days after transfection, the cells were split, seeded at a density of 100,000 cells per 10-cm dish, and grown in the presence of 0.8 mg of Geneticin G418 (GIBCO) per ml in the medium for neomycin resistance selection. Resistant clones were picked after 3 weeks, tested for expression of the CER by immunoprecipitation with antibody RK2 (9) and receptor self-phosphorylation, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Full details concerning the generation of NIH 3T3 cells that express the HER (HER14 cells) were previously published (6, 7).

Binding experiments. Cells were plated at a density of 100,000 cells per well in 24-well dishes coated with 20 μg of human plasma fibronectin (Meloy Laboratories) per well and allowed to grow for 48 h to confluency in DMEM containing 10% fetal bovine serum. Confluent cells in 24-well Costar dishes were washed with DMEM containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4) and 2 mg of bovine serum albumin per ml and then incubated in duplicate with [^{125}I]TGF-α (0.02 to 500 ng/ml; specific activity, about 100,000 cpm/ng) in the same buffer. Nonspecific binding was determined by parallel experiments of binding to parental untransfected cells (NIH 3T3 clone 2.2). After incubation for either 90 min at 4°C or 60 min at 24°C, the cells were placed on ice and washed four times with ice-cold DMEM containing 1 mg of bovine serum albumin. The cells were lysed in 1 ml of 1 M NaOH for 30 min at 37°C, and the radioactivity in the lysate was measured to determine the amount of bound ligand. A similar protocol was used for the binding of [^{125}I]EGF to cells that express the HER (6, 7). A computer program developed by Stephen Felder was used to fit the data to either a one- or a two-site model of ligand binding and to obtain receptor numbers and K_d values.

[^{35}S]methionine labeling. Subconfluent cells in 10-cm dishes were washed with methionine-free DMEM and grown for 12 h in methionine-free DMEM–10% fetal bovine serum containing 50 μCi of [^{35}S]methionine per ml. The cells were washed three times with DMEM, scraped into 0.5 ml of lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid], 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 mM phenylanethylsulfonyl fluoride), and incubated for 30 min on ice, and the lysate was spun for 5 min in an Eppendorf centrifuge in the cold. Protein A-Sepharose (3 mg per sample) was suspended in 20 mM HEPES (pH 7.5), washed with 20 mM HEPES, and incubated for 30 min at room temperature with antiserum RK2. The protein A-Sepharose-antibody complex was washed three times with HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). The immunoprecipitate was then washed once with 50 mM HEPES (pH 8.0)–0.2% Triton X-100–500 mM NaCl–5 mM EGTA, twice with 50 mM HEPES (pH 8.0)–0.1% Triton X-100–0.1% SDS–150 mM NaCl–5 mM EGTA, and once with 10 mM Tris hydrochloride (pH 8.0)–0.1% Triton X-100, and then 3 volumes of sample buffer was added to the washed immunoprecipitate, boiled for 4 min, and electrophoretically separated on a 7% SDS-polyacrylamide gel.

Phosphorylation experiments. In vitro autophosphorylation experiments were performed essentially as previously described (6, 7). Ligand-induced phosphorylation of the EGF-R in living cells was performed as follows. Confluent cells in six-well Costar (Cambridge, Mass.) dishes were starved with 0.5% serum for 12 to 16 h. Cells were incubated with EGF or TGF-α (1 or 10 μg/ml) for 20 min at 37°C, the medium was aspirated, and the cells were washed twice with cold phosphate-buffered saline and then solubilized with 50 μl of boiled sample buffer. The solubilized cells were heated at 95°C for 5 min and cooled to room temperature for 15 min. The samples were sonicated for 30 s and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 7% acrylamide. Subsequently, the gels were blotted onto nitrocellulose filters with Omniblot (ABN). The nitrocellulose blots were treated with 3% bovine serum albumin in phosphate-buffered saline overnight in the cold and then incubated for 4 h with antiphosphotyrosine antibodies (a gift from P. Comoglio), washed once with 10 mM Tris hydrochloride (pH 7.5)–150 mM NaCl (TBS), twice with TBS containing 0.1% Triton-X-100, and once with TBS (3). The washed filter was incubated for 1 h with ^{125}I-labeled protein A (200,000 cpm/ml) in TBS containing 3% bovine serum albumin and washed with the TBS solutions described above. The filters were autoradiographed for 1 to 3 days.

[^{3}H]thymidine incorporation. Cell lines expressing either the CER or the HER were seeded in 24-well Costar dishes at 10^5 cells per well and grown for 2 days. The cells were then incubated with medium supplemented with 0.5% fetal bovine serum containing either EGF or TGF-α (TGF-α, 0.05 to 100 ng/ml; EGF, 0.1 to 1 μg/ml). The cells were incubated for 18 h and [^{3}H]thymidine (5 μCi per well) was added for 4 h at 37°C. The cells were then washed, and the amount of trichloroacetic acid (5%)-precipitable radioactivity was determined. For chicken embryo fibroblasts, [^{3}H]thymidine incorporation was determined essentially as described by Betsholtz and Westermark (1).

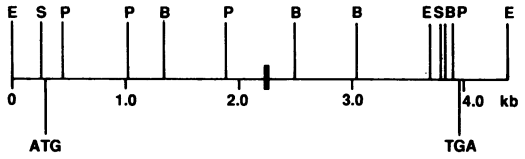

FIG. 1. Schematic diagram of CER cDNA. The beginning (ATG) and end (TGA) of the open reading frame are shown. The transmembrane region is marked by a heavy vertical line. Restriction sites: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I.

RESULTS

Isolation and sequence of CER cDNA. To isolate CER cDNA, a chicken embryo cDNA library in λgt11 (19) was screened with a v-*erbB* probe derived from the avian erythroblastosis virus genome (25). Ten positive plaques were isolated and tested for hybridization with a probe representing the extracellular portion of the HER (23). One of the isolated clones (C1.12), which contained two *Eco*RI fragments of 3.7 and 0.7 kb and showed strong hybridization with HER cDNA, was chosen for further analysis. The partial restriction enzyme map of C1.12 is shown in Fig. 1, and the sequence of the 5′ half of the cDNA is given in Fig. 2. The rest of the sequence was found to be identical to the sequence of c-*erbB* published by Nilsen et al. (15). C1.12 contains 290 base pairs of the 5′ nontranslated region and 450 base pairs of the 3′ nontranslated sequence. From the deduced amino acid sequence (Fig. 1; reference 15), we conclude that the CER gene encodes 1,223 amino acids, 13 more than the HER gene, 6 of which are in the signal sequence. The overall similarity between the CER and the HER is depicted in Fig. 3. The signal peptide of the CER is 6 residues longer than that of the HER. The extracellular

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CGGCTGGCCCCGGCTGCTCCGCAAACCCTGTGACAGAAAGGAGCGCAGGGAGGAGGAAAGAAGAGGAGGAGAAGGAAGGGGAGGAGGAAGAAGAAGAGGAAGAAGGCAGCAGTCCGCCCGCAGCTGGGTAGGCACGTGTGCGCGCAGCCC  150

                                                                                                                                                    -30
                                                                                                                                                   MetGlyVal
TCGACGGCCGCCCTCCAGCATGCTGCGCGGAGGCAGAGCGGCAGCTCGGAGCTGAGCGGGGTGGAGCGGAGCGCAGAGCAGTGCAGAGTGAGGAGCAGCTCGTCGGGGAGCTCCGTGTGCCCGTCGCGTCCGACGCACACCATGGGTGTA  300

                -20                 -10                  1                  10                  20
ArgSerProLeuSerAlaSerGlyProArgGlyAlaAlaValLeuValLeuLeuLeuLeuGlyValAlaLeuCysSerAlaValGluGluLysLysValCysGlnGlyThrAsnAsnLysLeuThrGlnLeuGlyHisValGluAspHis
CGCAGCCCCCTGTCCGCCTCTGGGCCTCGCGGGGCCGCTGTCCTGGTGCTACTGCTGCTGGGCGTCGCGCTGTGTTCCGCCGTGGAGGAGAAGAAAGTTTGTCAAGGGACAAATAACAAGTTGACCCAGCTGGGGCACGTGGAAGACCAT  450

        30                  40                  50                  60                  70
PheThrSerLeuGlnArgMetTyrAsnAsnCysGluValValLeuSerAsnLeuGluIleThrTyrValGluHisAsnArgAspLeuThrPheLeuLysThrIleGlnGluValAlaGlyTyrValLeuIleAlaLeuAsnMetValAsp
TTCACCAGCCTGCAGAGAATGTACAACAACTGCGAAGTGGTACTGAGTAACCTGGAGATTACCTACGTGGAGCATAATCGCGATCTCACCTTCCTTAAGACCATACAGGAGGTTGCAGGCTATGTGCTCATTGCGCTTAACATGGTGGAC  600

        80                  90                  100                 110                 120
ValIleProLeuGluAsnLeuGlnIleIleArgGlyAsnValLeuTyrAspAsnSerPheAlaLeuAlaValLeuSerAsnTyrHisMetAsnLysThrGlnGlyLeuArgGluLeuProMetLysArgLeuSerGluIleLeuAsnGly
GTCATTCCCTTAGAAAACCTCCAGATTATCCGAGGGAATGTGCTTTATGACAACTCTTTTGCCCTGGCAGTTTTATCCAATTACCACATGAATAAAACCCAGGGACTTCGAGAGCTGCCAATGAAACGGCTATCAGAAATTCTCAATGGA  750

        130                 140                 150                 160                 170
GlyValLysIleSerAsnAsnProLysLeuCysAsnMetAspThrValLeuTrpAsnAspIleIleAspThrSerArgLysProLeuThrValLeuAspPheAlaSerAsnLeuSerSerCysProLysCysHisProAsnCysThrGlu
GGTGTTAAAATCAGCAACAACCCCAAACTGTGCAACATGGACACTGTTCTCTGGAATGACATCATTGATACAAGCAGGAAGCCTCTCACAGTACTTGACTTTGCAAGCAATCTTTCTTCTTGTCCAAAATGCCATCCGAACTGCACAGAA  900

        180                 190                 200                 210                 220
AspHisCysTrpGlyAlaGlyGluGlnAsnCysGlnThrLeuThrLysValIleCysAlaGlnGlnCysSerGlyArgCysArgGlyLysValProSerAspCysCysHisAsnGlnCysAlaAlaGlyCysThrGlyProArgGluSer
GACCACTGCTGGGGTGCTGGTGAACAGAACTGCCAGACTTTAACAAAAGTCATCTGTGCCCAGCAATGCTCTGGCCGGTGCAGAGGAAAGGTGCCCAGTGACTGCTGCCACAATCAGTGTGCTGCAGGGTGCACAGGACCTCGGGAGAGT 1050

        230                 240                 250                 260                 270
AspCysLeuAlaCysArgLysPheArgAspAspAlaThrCysLysAspThrCysProProLeuValLeuTyrAsnProThrThrTyrGlnMetAspValAsnProGluGlyLysTyrSerPheGlyAlaThrCysValArgGluCysPro
GACTGCCTGGCATGCCGCAAGTTTCGGGATGATGCTACCTGCAAGGACACATGTCCCCCACTGGTCCTCTATAACCCCACCACCTATCAAATGGATGTCAACCCTGAGGGAAAATACAGCTTTGGAGCCACTTGTGTGAGGGAATGTCCA 1200

        280                 290                 300                 310                 320
HisAsnTyrValValThrAspHisGlySerCysValArgSerCysAsnThrAspThrTyrGluValGluGluAsnGlyValArgLysCysLysLysCysAspGlyLeuCysSerLysValCysAsnGlyIleGlyIleGlyGluLeuLys
CACAACTATGTGGTGACAGATCATGGCTCCTGCGTTCGCTCGTGTAATACTGATACTTACGAAGTGGAAGAAAATGGTGTTCGGAAGTGTAAAAAATGTGATGGGCTATGTAGCAAAGTGTGCAATGGCATTGGAATAGGTGAACTTAAA 1350

        330                 340                 350                 360                 370
GlyIleLeuSerIleAsnAlaThrAsnIleAspSerPheLysAsnCysThrLysIleAsnGlyAspValSerIleLeuProValAlaPheLeuGlyAspAlaPheThrLysThrLeuProLeuAspProLysLysLeuAspValPheArg
GGGATCCTATCCATAAATGCCACAAACATCGACTCCTTCAAAAACTGTACGAAGATCAATGGGGATGTCAGCATTCTTCCTGTTGCATTTCTAGGGGATGCCTTCACAAAGACACTACCCCTTGACCCTAAGAAGCTGGATGTCTTTAGA 1500

        380                 390                 400                 410                 420
ThrValLysGluIleSerGlyPheLeuLeuIleGlnAlaTrpProAspAsnArgThrAspLeuTyrAlaPheGluAsnLeuGluIleIleArgGlyArgThrLysGlnHisGlyGlnTyrSerLeuAlaValAlaAsnLeuLysIleGln
ACAGTCAAAGAAATATCAGGATTTTTGTTGATTCAGGCCTGGCCTGATAATGCTACTGATCTCTATGCTTTTGAAAATCTGGAGATTATCCGAGGCCGAACCAAGCAGCACGGCCAGTATTCCCTTGCTGTTGTTAACTTGAAAATACAG 1650

        430                 440                 450                 460                 470
SerLeuGlyLeuArgSerLeuLysGluIleSerAspGlyAspIleAlaIleMetLysAsnLysAsnLeuCysTyrAlaAspThrMetAsnTrpArgSerLeuPheAlaThrGlnSerGlnLysThrLysIleIleGlnAsnArgAsnLys
TCGTTGGGGCTGCGCTCCCTCAAGGAAATAAGTGATGGAGACATTGCCATTATGAAGAACAAGAACCTCTGCTATGCTGACACCATGAACTGGCGCAGCTTGTTTGCTACTCAGAGTCAGAAAACAAAAATTATACAGAACAGAAATAAA 1800

        480                 490                 500                 510                 520
AsnAspCysThrAlaAspArgHisValCysAspProLeuCysSerAspValGlyCysTrpGlyProGlyProHisCysPheSerCysArgPhePheSerArgGlnLysGluCysValLysGlnCysAsnIleLeuGlnGlyGluPro
AATGATTGTACTGCTGACAGGCATGTGTGTGACCCGCTGTGCTCGGACGTGGGCTGCTGGGGCCCAGGGCCCTTCCACTGCTTTTCCTGCAGGTTTTTCAGTCGCCAGAAGGAGTGTGTAAAACAGTGCAACATCCTGCAAGGGGAGCCA 1950

        530                 540                 550                 560                 570
ArgGluPheGluArgAspSerLysCysLeuProCysHisSerGluCysLeuValGlnAsnSerThrAlaTyrAsnThrThrCysSerGlyProGlyProAspHisCysMetLysCysAlaHisPheIleAspGlyProHisCysValLys
CGTGAGTTTGAAAGAGACTCCAAATGCCTCCCCTGCCACTCAGAGTGTCTGGTACAGAACTCCACTGCATACAACACAACCTGCTCTGGACCGGGCCCAGACCACTGCATGAAGTGTGCCCATTTTATAGATGGTCCCCACTGTGTGAAG 2100

        580                 590                 600                 610                 620
AlaCysProAlaGlyValLeuGlyGluAsnAspThrLeuValTrpLysTyrAlaAspAlaAsnAlaValCysGlnLeuCysHisProAsnCysThrArgGlyCysLysGlyProGlyLeuGluGlyCysProAsnGlySerLysThrPro
GCCTGCCCCGCTGGGGTCCTGGGTGAGAATGATACCCTGGTCTGGAAGTATGCAGATGCCAATGCTGTTTGCCAGCTCTGCCATCCAAACTGTACACGAGGGTGCAAAGGGCCAGGTCTTGAAGGATGTCCAAATGGCTCCAAAACTCCA 2250

        630                 640                 650                 660                 670
SerIleAlaAlaGlyValValGlyGlyLeuLeuCysLeuValValValGlyLeuGlyIleGlyLeuTyrLeuArgArgArgHisIleValArgLysArgThrLeuArgArgLeuLeuGlnGluArgGluLeuValGluProLeuThrPro
TCTATCGCGGCTGGTGTTGTCGGAGGACTCCTGTGCCTGGTTGTGGTTGGTCTAGGCATCGGTCTTTACCTGCGGCGACGTCATATCGTGCGGAAGCGCACCCTGCGCAGGCTGCTGCAAGAGAGGGAGCTTGTCGAACCACTGACACCC 2400
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FIG. 2. Nucleotide sequence and deduced amino acid sequence of the 5′ half of CER cDNA. The sequence was determined by subcloning of *Eco*RI-*Pst*I; *Pst*I, and *Bam*HI-*Pst*I fragments of the cDNA (Fig. 1) into M13 and sequencing by the dideoxy chain termination method (18). Sequences overlapping the restriction sites were determined with synthetic oligonucleotides as primers. The sequence was determined on both strands for the 5′ half of the cDNA (to the transmembrane region), and for the 3′ half the sequence was determined only for several segments, confirming the previously published sequence (15). The sequence of c-*erbB* published by Nilsen et al. (15) begins at nucleotide 2041 (codon 555) in the sequence shown. The cDNA described in Fig. 1 ends at nucleotide 2847 of the sequence of c-*erbB* (15) and does not include the polyA signal. The transmembrane region is underlined. The numbers at the right indicate nucleotides, and the numbers above the lines indicate amino acids.

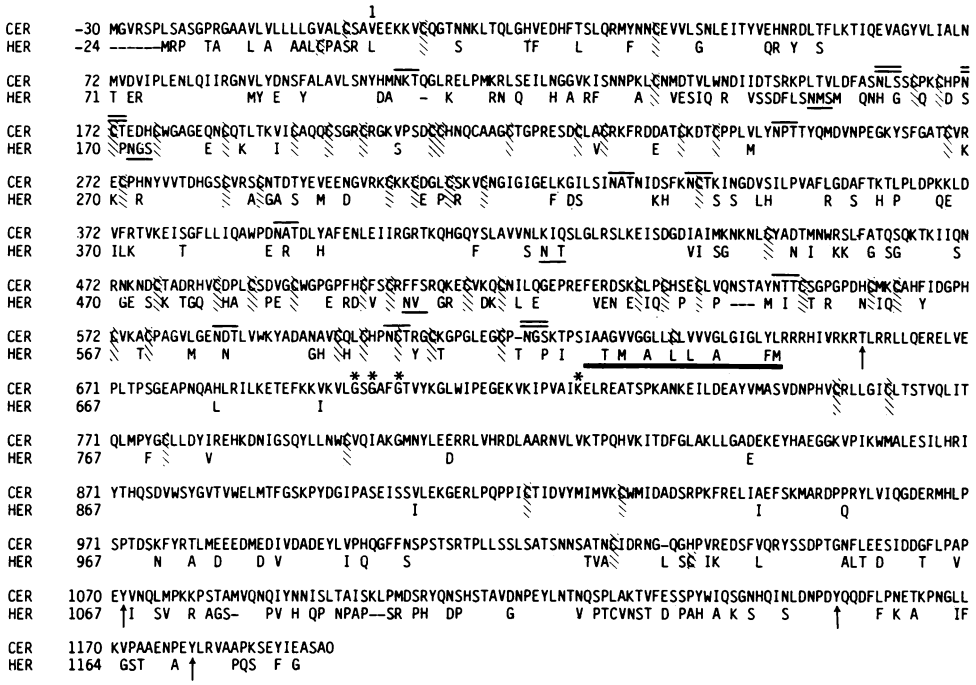

FIG. 3. Comparison of the deduced amino acid sequences of the CER and the HER. The transmembrane region is heavily underlined. Blanks represent identical residues. Dashes represent deletions. Cysteine residues are hatched. Phosphorylated residues are marked by arrows, and residues conserved in the ATP-binding site are marked by asterisks. Potential N-glycosylation sites are underlined (CER) or overlined (CER). Conserved N-glycosylation sites are marked by single lines, and different N-glycosylation sites are marked by double lines. The data are from Fig. 2 and reference 15.

portions of the CER and the HER show 75% identical residues. The cytoplasmic domains show 97% identity in the tyrosine kinase domain and only 65% identity in the C-terminal 227 residues. All cysteine residues of the two extracellular cysteine-rich domains, as well as the major phosphorylation sites corresponding to residues Thr-654, Tyr-1068, Tyr-1148, and Tyr-1173 of the HER are conserved. The extracellular portion contains 11 potential N-linked glycosylation sites, 8 of which are identical to those present in the HER and 2 others of which are in very close proximity to similar sites in the HER.

Inspection of the amino acid sequence of the extracellular domains of the CER reveals an organization similar to that of the HER. The 622 amino acids of the extracellular portion can be subdivided into four domains as follows: domain I, residues 1 to 160; domain II, residues 161 to 310, which contains 24 Cys residues and is the first Cys-rich domain; domain III, residues 311 to 475; domain IV, residues 476 to 620, which contains 20 Cys residues and is denoted the second Cys-rich domain. To analyze further the evolutionary relationship between different segments of the extracellular portion of the EGF-R, we compared the amino acid sequences of domains I and III. Figure 4 depicts the similarity between domains I and III of the CER and the HER and indicates that the two domains may have evolved by gene duplication. Each of these segments contain two cysteines separated by 100 (domain I) or 108 (domain III) residues which may form an intradomain disulfide bond. If Cys-34 and Cys-134 (domain I) are aligned with Cys-339 and Cys-447 (domain III), the sequences of the two domains show 40 identical residues (33.5%). Most of these residues are also conserved in domains I and III of the HER. Since the two cysteine-rich domains (II and IV) also show high conservation of the cysteine residues, it is possible that the unit of gene duplication was composed of domains I and II together, which duplicated to form domains III and IV (22).

Expression of the CER in murine cells. To compare the binding properties and receptor function of the CER with those of the HER, we inserted the CER into the pLSV vector that was used previously for expression of the HER in NIH 3T3 and CHO cells (12, 13). The 4.4-kb *Xho*I fragment, containing the entire CER cDNA was subcloned into the *Xho*I site of the modified pLSV (12) to generate pLSV which contains the CER in an orientation that juxtaposes the 5′ end to the simian virus 40 promoter region and the 3′ end to the simian virus 40 polyadenylation site (see Materials and Methods).

This plasmid was cotransfected into NIH 3T3 cells with pSVNeo, followed by selection with Geneticin (G-418). Resistant-cell clones were analyzed for expression of the CER as described in Materials and Methods. Several anti-HER antibodies were used to precipitate the in vivo

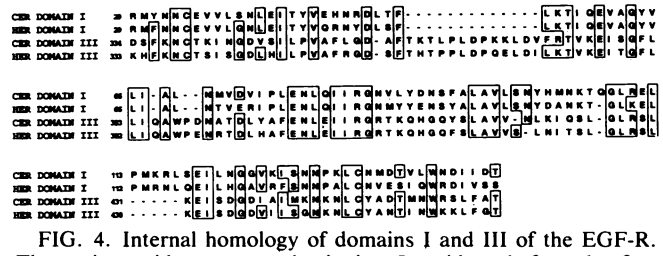

FIG. 4. Internal homology of domains I and III of the EGF-R. The amino acid sequences beginning 5 residues before the first cysteines and ending 13 residues after the second cysteines of domains I and III (see Fig. 10) were aligned with a minimal number of gaps. Identical residues are boxed. The numbers to the left correspond to the sequences shown in Fig. 3.

[^{35}S]methionine-labeled CER in these cell lines. Polyclonal RK2 antibodies (9), directed against a synthetic peptide of residues 984 to 996 of the HER, and antibodies, directed against a synthetic peptide of residues 659 to 667 or the HER (F. Bellot et al., manuscript in preparation), precipitated the CER efficiently. Figure 5 (lanes A to D) shows the immunoprecipitated [^{35}S]methionine-labeled CER from four cell lines (CER83, CER110, CER76, and CER109) compared with that of HER14 (lane F), which expresses the HER (6, 7). The apparent molecular mass of the CER is 170 kilodaltons, which is indistinguishable from that of the HER. Figure 5 also shows that the parental NIH 3T3 line (clone 2.2) did not contain detectable EGF-R (lane E). Furthermore, the transfected cells expressing the CER showed no trace of endogenous mouse EGF-R by immunoprecipitation with an antibody to the C-terminal peptide of the HER, which also cross-reacts with the mouse EGF-R (Bellot et al., in preparation) but does not immunoprecipitate the CER.

Differential binding of TGF-α and EGF to the CER. The HER binds two different growth factors equally well: EGF and TGF-α (21). We therefore compared the parameters of binding of the CER in clone CER109 to mouse EGF and human TGF-α. Binding experiments with ^{125}I-labeled mouse EGF were difficult to interpret because of the high nonspecific binding (60%) observed with the high concentration of EGF (5 μg/ml) used in the binding experiment. On the other hand, the affinity of the CER to TGF-α was found to be comparable to that of the HER. We therefore determined by Scatchard analysis the binding parameters of the CER to TGF-α and then analyzed the dissociation constant of EGF by displacement of labeled TGF-α. The affinity of TGF-α for the CER (K_d, 0.7 nM) was slightly higher than its affinity for the HER (K_d, 1.5 nM) (Fig. 6), when the receptors were expressed in NIH 3T3 cells (clones CER109 and HER14, respectively). Although this difference was not large, it was reproduced in three different experiments. The binding data also indicated a single affinity constant of TGF-α for both receptors. This was in contrast to EGF, which bound to the

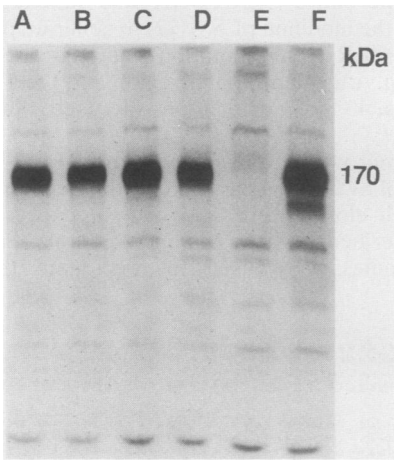

FIG. 5. Identification of the CER and the HER by immunoprecipitation of [^{35}S]methionine-labeled cells. Cells from isolated clones were labeled with [^{35}S]methionine, lysed, and immunoprecipitated with RK2 anti-EGF-R antibodies. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with a 7% polyacrylamide gel, and autoradiography. The lanes contained NIH 3T3 cells expressing the CER clones CER110 (A), CER83 (B), CER109 (C), and CER76 (D); parental NIH 3T3 cells, NIH 3T3 2.2 cells (E); and NIH 3T3 cells expressing HER, HER14 cells (F). kDa, Kilodaltons.

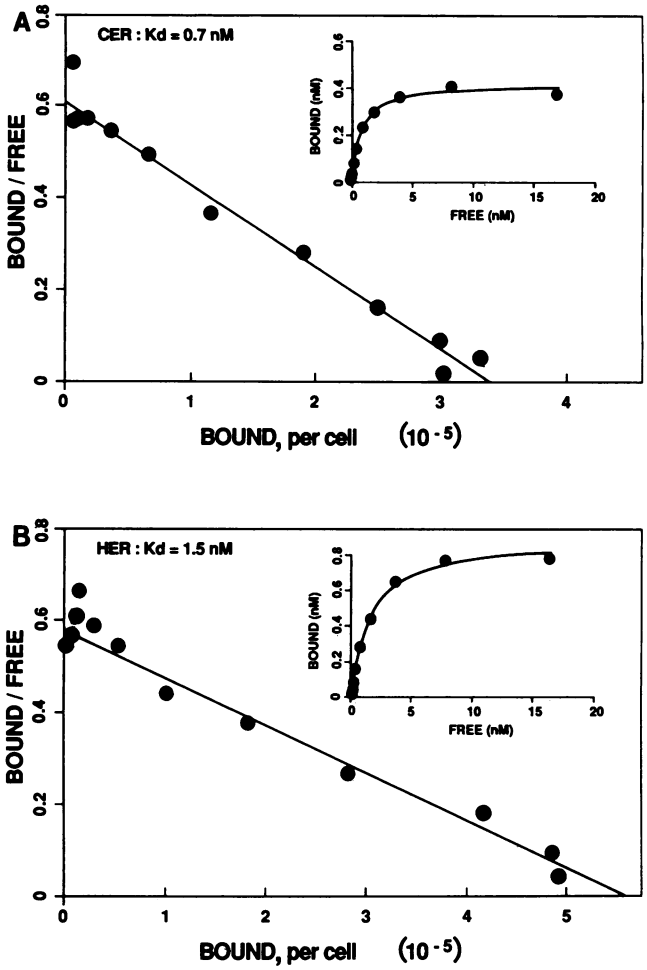

FIG. 6. Binding of [^{125}I]TGF-α to cells expressing either the HER or the CER. [^{125}I]TGF-α binding to cells expressing either the CER (CER109 cells) or the HER (HER14 cells) was determined as detailed in Materials and Methods. The binding curves from duplicate measurements are presented as Scatchard plots determined by a computer program that fit the data to either a one- or a two-site model of ligand binding. This experiment was repeated three times with essentially the same results. A, CER; B, HER.

HER with two affinities: high affinity (K_d, 5×10^{-10} M) and low affinity (K_d, 15×10^{-9} M) (reviewed in reference 20 and D. Schlessinger, in press). The single-binding affinity for TGF-α fell in the range between the high- and low-affinity K_d values measured for EGF (Table 1). We also determined the binding of human TGF-α to chicken embryo fibroblasts. The Scatchard plot showed a single affinity, with a K_d of 0.65 nM. This value is similar to the affinity measured for the CER (Table 1), indicating that the cloned CER expressed in NIH 3T3 cells is the authentic CER. Binding of ^{125}I-labeled mouse EGF to chicken embryo fibroblasts could not be measured because of the low affinity of mouse EGF for the CER and the low expression of EGF-R in chicken cells.

To determine the K_d of EGF for the CER or the HER, we analyzed displacement of labeled TGF-α by unlabeled EGF under conditions in which about 5 to 10% of the sites of the receptor were occupied by TGF-α. Figure 7 and Table 1 show the results of this analysis and indicate that the K_d of EGF for the HER was 2.8 nM and that of EGF for the CER was 140 nM. The data for the HER are consistent with the K_d obtained from direct measurement of EGF binding to the

TABLE 1. Dissociation constants of the CER and the HER for EGF and TGF-α

Receptor	Growth factor	K_d (10^{-9} M)
HER in mouse cells	EGF	0.5 (5%); 15 (95%)[a]
	TGF-α	1.5
CER in mouse cells	EGF	140[a]
	TGF-α	0.7
CER in chicken embryo fibroblasts	TGF-α	0.65

[a] The K_d of EGF for the CER was estimated from [^{125}I]TGF-α displacement experiments (Fig. 7). This analysis yielded a K_d of 2.8×10^{-9} M for the binding of EGF to the HER expressed in mouse cells. However, direct [^{125}I]EGF-binding experiments revealed typical high (5%)- and low (95%)-affinity binding sites for the HER.

HER (reviewed in reference 20 and J. Schlessinger, in press) and suggest that EGF and TGF-α compete for the same site on the EGF-R. However, direct binding experiments revealed an additional high-affinity state (Table 1) which comprises 5% of the total receptors. Taken together, the results of Fig. 6 and 7 indicate that the HER bound TGF-α and EGF at comparable affinities, whereas the affinity of the CER for TGF-α was about 200-fold higher than that for EGF (0.7 nM compared with 140 nM). The CER and the HER have similar affinities to TGF-α, whereas the affinity of EGF to the CER is 50-fold less than its affinity for the HER. These results demonstrate differential binding of the CER to the two mammalian ligands EGF and TGF-α although both growth factors compete for the same binding site.

Functional analysis of the CER in murine cells. Ligand binding by the extracellular portion of the EGF-R leads to activation of the cytoplasmic protein tyrosine kinase domain and initiation of pleiotropic responses, leading to DNA synthesis and cell proliferation. We wanted to measure whether the CER, expressed in NIH3T3 cells, is able to mediate these responses.

We analyzed the kinase activity of the CER and its enhancement by either EGF or TGF-α by measuring the extent of self-phosphorylation of the receptor in vitro and in living cells. Lysates prepared from CER109 or HER14 cells were first incubated with the ligands or with buffer alone, and then the EGF-R was immunoprecipitated with RK-2 antibodies attached to Sepharose beads (9). The washed immunoprecipitates were subjected, for 1 min at room temperature, to a phosphorylation reaction mixture containing [γ-^{32}P]ATP, leading to self-phosphorylation. Immunoprecipitates were electrophoresed on 7% polyacrylamide gel, followed by autoradiography and counting of the radioactive content of the EGF-R band. Self-phosphorylation of the HER was similarly enhanced by EGF and TGF-α (5- to 10-fold; Fig. 8A). Self-phosphorylation of the CER was likewise enhanced 5- to 10-fold by TGF-α, while a higher concentration of EGF was required for similar stimulation. This result probably reflects the lower binding affinity of EGF than TGF-α for the CER. In addition, we applied phosphotyrosine antibodies to demonstrate that EGF and

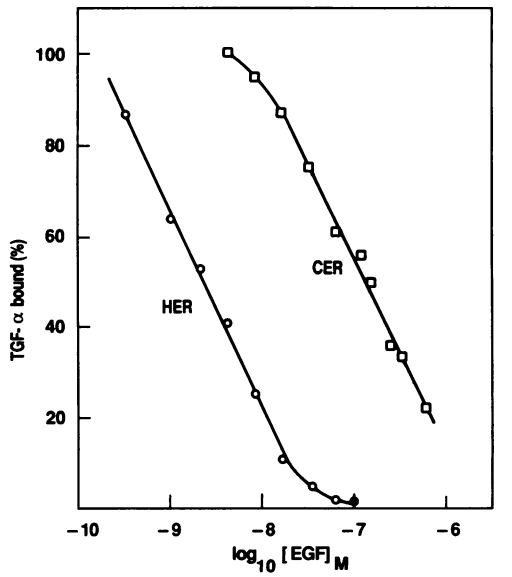

FIG. 7. Inhibition by EGF of binding of ^{125}I-labeled TGF-α to cells expressing either the HER or the CER. EGF at increasing concentrations was used to displace [^{125}I]TGF-α at a constant concentration from cells expressing either the HER (HER14 cells) or the CER (CER109 cells). The cells were incubated with a concentration of [^{125}I]TGF-α which saturates approximately 5% of the EGF receptors. The concentration of EGF was varied for the HER from 1 ng/ml to 1 μg/ml, and for the CER it was varied from 25 ng/ml to 4 μg/ml.

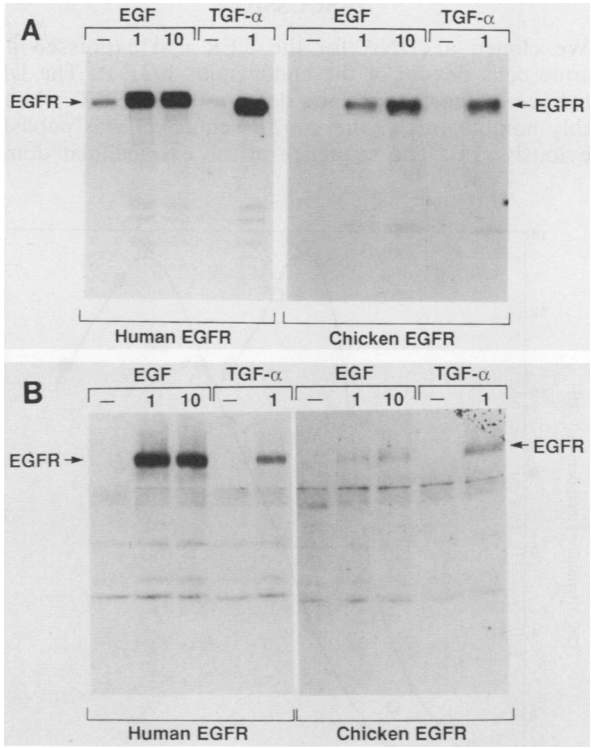

FIG. 8. Autophosphorylation of the CER and the HER in vitro and in living cells. The effects of TGF-α and EGF on autophosphorylation of the CER and the HER were analyzed. (A) NIH 3T3 cells expressing either the CER (CER109 cells) or the HER (HER14 cells) were incubated in the absence or presence of either EGF or TGF-α and then immunoprecipitated with RK2 antibodies. The immunoprecipitates were subjected to the standard conditions of the phosphorylation reactions. (B) Cells expressing the CER (CER109 cells) or the HER (HER14 cells) were exposed to either TGF-α or EGF for 20 min, followed by cell solubilization, electrophoretic separation on gels, electroblotting, and analysis with antiphosphotyrosine antibodies as described previously (3).

TGF-α stimulate autophosphorylation of the avian EGF-R and the HER in vivo (Fig. 8). As before, EGF and TGF-α similarly enhanced autophosphorylation of the HER, whereas a higher concentration of EGF was required for the CER.

To analyze the biological function of the CER in NIH 3T3 cells, we measured the effects of TGF-α and EGF on DNA synthesis in CER expression cells. Confluent cells were starved in 0.5% serum for 24 h, EGF or TGF-α was added at various concentrations. After 18 h of incubation, [^{3}H]thymidine (1 μCi per well) was added and the incorporated [^{3}H]thymidine was measured after 4 h. Both TGF-α and EGF stimulated DNA synthesis in CER cells to similar extents (Fig. 9). However, maximal stimulation by TGF-α was at 5 ng/ml, whereas maximal stimulation of EGF was at 500 ng/ml (Fig. 9 and Table 2). This 100-fold difference in the concentration of EGF required for optimal stimulation of cell proliferation is in accord with the approximately 100-fold difference between the binding affinities of the two ligands for the CER. In chicken embryo fibroblasts, maximal stimulation of DNA synthesis by human TGF-α and mouse EGF was with 1 and 100 ng/ml, respectively. This is consistent with the results obtained with transfected NIH 3T3 cells, indicating that the ligand specificity of the CER is similar when expressed in chicken cells or transfected murine cells.

DISCUSSION

We cloned a cDNA for the CER and expressed it in murine cells devoid of the endogenous EGF-R. The DNA sequence of the cytoplasmic domain of the CER, which is highly homologous to the v-*erbB* sequence, was published previously (15). The sequence of the extracellular domain shows remarkable homology to the human EGF-R (Fig. 10). All of the cysteine residues and most of the potential N-glycosylation sites in the extracellular portion are conserved. In addition, the extracellular portion was subdivided into four domains (I to IV, Fig. 10); domains II and IV are the cysteine-rich domains. By aligning the sequences of domains I and III, we showed that these domains probably represent homologous repeats for both the HER and the CER. Figure 4 shows 27% identical residues in both the HER and the CER in domains I and III. If positions where three of four residues are identical (Fig. 4) are included in the comparison, the similarity is 37%, and if we also include conservative replacements, the similarity is 49%. Domains I and III contain two cysteine residues which can form an intradomain disulfide bond, allowing independent folding of each domain. We assume also that the cysteine-rich segments (II and IV) may fold as independent domains. On the basis of this internal homology and other studies, we propose a four-domain model for the extracellular portion of the EGF-R. Moreover, on the basis of affinity labeling of the EGF-R with EGF (10) and the ligand-binding properties of a deletion mutant devoid of domain I (I. Lax, F. Bellot, A. M. Honeggar, A. Schmidt, A. Ullrich, D. Givol, and J. Schlessinger, submitted for publication), we suggested that domain III contributes most of the forces which define the ligand-binding specificity of the EGF-R (10).

One of the aims of this study was to compare the binding properties of the CER and the HER. It was anticipated that mouse EGF would bind to the CER with a lower affinity than to the HER. Indeed, the binding affinity of EGF to the CER was 100-fold lower than its affinity to the HER. However,

TABLE 2. Stimulation of DNA synthesis

Cells	Growth factor	Stimulation ratio[a]	Maximal stimulation by growth factor (ng/ml)
HER in NIH 3T3	EGF	10.5	100
	TGF-α	6.0	50
CER in NIH 3T3	EGF	12.0	500
	TGF-α	12.5	5
Chicken embryo fibroblasts	TGF-α	1.6[b]	1

[a] The stimulation ratio is the maximal [^{3}H]thymidine counts per minute incorporated in the presence of growth factor divided by the incorporated counts per minute in the absence of growth factor.

[b] The small stimulation obtained with chicken embryo fibroblasts was due to the high basal stimulation detected in these cells, which could not be brought to a quiescent state, probably because they produce their own growth factors.

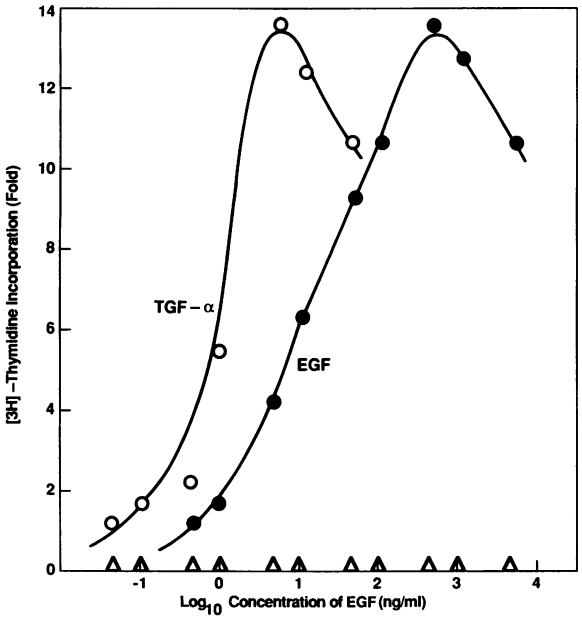

FIG. 9. Stimulation of DNA synthesis by either TGF-α or EGF. Cells expressing the CER (CER109 cells) were brought to quiescence and stimulated with various amounts of EGF or TGF-α. After 18 h [^{3}H]thymidine was added for 4 h and the trichloroacetic acid-precipitable radioactivity was determined. The counts per minute in the absence of growth factor (basal) were 4,043 for the CER and 9,510 for the HER. For 1% serum-stimulated cells, the counts per minute were 20,500 for the CER and 65,870 for the HER.

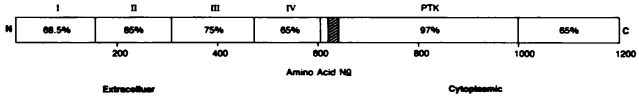

FIG. 10. Similarity between the amino acid sequences of the HER and the CER. The extracellular portion was subdivided into four domains; domains II and IV are the cysteine-rich regions (see the text). The numbers were calculated from the data in Fig. 3. PTK, Protein tyrosine kinase domain.

the finding (Fig. 5) that human TGF-α binds to the CER with higher affinity (K_d, 0.7 nM) than to the HER (K_d, 1.5 nM) was unexpected. It would be plausible to assume that because of the evolutionary distance between the avian receptor and the mammalian growth factor, the binding affinity for both ligands (EGF and TGF-α) is similarly reduced. This, however, is not the case and raises several interesting evolutionary and functional problems.

The structural basis for the comparable binding of EGF and TGF-α to the HER is not clear. The sequence identity between mouse EGF and human TGF-α is only 32%, but perhaps the configurational similarity of the three disulfide loops contributes significantly to their similar functions in binding and activating the EGF-R. The availability of the avian EGF-R and HER cDNAS will allow the design of in vitro domain exchange and characterization of the differences between the binding of TGF-α and EGF to the two receptors, enabling localization of the EGF-binding site.

From the binding and mitogenic properties of human TGF-α it is possible that this growth factor can function as a physiological growth factor in birds, since its binding affinities for the CER and the HER are similar. Moreover, functionally, the v-*erbB* oncogene which was transduced by an avian retrovirus (avian erythroblastosis virus) may in fact be regarded as a truncated TGF-α receptor rather than a truncated EGF-R (5, 27). On the other hand, EGF can be physiological growth factor in birds only if a chicken EGF has a significantly higher affinity to the receptor than that found for murine EGF. However, there is no information concerning the structure or even the existence and tissue distribution of EGF and TGF-α in birds. One plausible explanation for the differential binding of EGF and TGF-α to the CER is that the divergence of TGF-α from birds to mammals is much less extensive than that of EGF. Yet another possibility is that TGF-α is indeed the original growth factor for the EGF-R, whereas EGF evolved later. The analysis of these possibilities must await the characterization of these growth factors and their genes in birds.

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