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Identification of the Minimal Requirements for Binding to the Human Epidermal Growth Factor (EGF) Receptor Using Chimeras of Human EGF and an EGF Repeat of *Drosophila* Notch*

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Many proteins contain so-called epidermal growth factor (EGF)-like domains that share the characteristic spacing of cysteines and glycines with members of the EGF family. They are, however, functionally unrelated, despite the fact that the three-dimensional structure of these EGF-like domains, also, is often very similar to that of the EGF receptor agonists. In the present study, we linked an EGF-like repeat from the *Drosophila* Notch protein to the N- and C-terminal linear tail sequences of human EGF (hEGF), and we showed that this chimera (E1N6E) is unable to bind or activate the hEGF receptor. This recombinant protein was then used as a basic construct for identifying the minimal requirements for high affinity EGF receptor binding and activation. We selectively reintroduced a limited number of important hEGF-derived residues, and by using this unique approach, we were able to make hEGF/Notch chimeras that, compared with wild type hEGF, showed nearly 100% binding affinity and mitogenic activity on HER-14 cells expressing the hEGF receptor.

Polypeptide growth factors acting through their tyrosine kinase receptors play a central role in the outgrowth of tumors. Many tumor cells are able to secrete such growth factors, to which they can respond in an autocrine fashion. High expression of members of the epidermal growth factor (EGF)[1] family, in particular of transforming growth factor α (TGFα), has been found in a variety of human cancers including breast and prostate cancers; oral and head and neck cancers; tumors of the gastrointestinal tract; and colon, lung, liver, kidney, and ovarian cancers (1–4). Because most of these tumors also express the EGF receptor, there is evidence that TGFα cause autocrine growth stimulation in these tumors. In order to interfere with such autocrine processes, it might be of great interest to develop an EGF receptor antagonist, an EGF-like protein with high receptor binding affinity that is, however, unable to activate the receptor. Although attempts to design such an antagonist have as yet been unsuccessful, much progress has been made in elucidating the requirements for interaction of EGF with its receptor.

There is much evidence to date that several residues in the so-called C-loop and in the C-terminal linear tail of EGF and TGFα are directly involved in high affinity binding to the EGF receptor. Site-directed mutagenesis studies have indicated that Arg^{41} and Leu^{47}, especially in hEGF, are highly conserved among the EGF receptor agonists and are irreplaceable (reviewed in Ref. 5). Furthermore, there is evidence that the C-terminal tail of TGFα, which is flexible in solution, becomes immobilized upon binding of TGFα to the hEGF receptor (6). Two-dimensional NMR studies have shown that Arg^{41} and Gln^{43} are in close contact with Tyr^{13}, Leu^{15}, and His^{16}, which indicates the possible existence of a nonlinear binding domain comprising residues from both the C- and N-terminal domains (6, 7). We have, in the past, constructed exchange mutants between hEGF and human TGFα and provided evidence that ARG^{45} also, in the C-terminal tail is part of the receptor binding pocket (8, 9). Using the same set of hEGF/human TGFα chimeras we have recently found that the 13A9 antibody, which specifically blocks the binding of human TGFα but not of hEGF, interferes with receptor binding of the C-terminal tail of human TGFα.[2] This again emphasizes the importance of this region in receptor recognition. The B-loop, at the opposite site of the molecule, is, however, much less conserved among EGF receptor agonists, and we have recently shown, using chimeras of hEGF and the *Drosophila* EGF receptor antagonist Argos, that several nonconservative substitutions can be made in the B-loop of hEGF without a significant effect on binding affinity (11).

Interestingly, a wide variety of proteins, including those involved in blood coagulation, neural development and cell adhesion, contain so-called EGF-like domains that share the characteristic spacing of six cysteines and three glycines with members of the EGF family but are functionally unrelated (12). Although they have no apparent binding affinity for the EGF receptor, increasing evidence exists that the three-dimensional structure of these EGF-like repeats is very similar to that of genuine EGF (13–17). Because a correct protein conformation is a prerequisite for high affinity receptor binding, this makes these EGF-like repeats interesting candidates for domain exchange studies.

In the present study, several chimeras were made between hEGF and EGF repeat 13 from Notch. The *Drosophila* notch gene encodes a 300-kDa transmembrane receptor with a large extracellular domain containing 36 EGF-like repeats (18), of which numbers 11 and 12, especially, are involved in binding of the ligands Delta and Serrate. Notch is widely expressed in the *Drosophila* embryo, and upon ligand binding, it controls cell fate in many tissues (for recent reviews, see Refs. 19 and 20).

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[1] The abbreviations used are: EGF, epidermal growth factor; hEGF, human EGF; mEGF, murine EGF; TGFα, transforming growth factor α; HPLC, high performance liquid chromatography; RP, reversed-phase; DMEM, Dulbecco's modified Eagle's medium; NCS, newborn calf serum; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

[2] A. E. G. Lenferink, A. D. G. Roos, M. J. H. Van Vugt, M. L. M. Van de Poll, and E. J. J. Van Zoelen, submitted for publication.

We selected EGF repeat 13 (amino acids 530–560) because of its relatively high sequence homology with hEGF and because it already contains several residues (*e.g.* Arg^{41}) that have been convincingly shown to be required for high affinity receptor binding. Despite this, the synthetic Notch EGF repeat was unable to bind the hEGF receptor. By selective reintroduction of hEGF-derived amino acids in to the Notch sequence, we were able to determine the minimal structural requirements for hEGF receptor binding. Using this unique approach, a functionally unrelated inactive EGF-like sequence was turned into a bioactive hEGF receptor ligand by introduction of only a limited number of important EGF-derived residues.

EXPERIMENTAL PROCEDURES

DNA Constructs—The synthetic gene for hEGF (British Biotechnology, Oxford, United Kingdom) was linked at the 5′-end to the sequence coding for the recognition sequence (Ile-Glu-Gly-Arg) of the proteolytic enzyme factor X (21). The resulting *Bam*HI-*Sal*I fragment (FX/EGF) was then placed in frame 3′ of the sequence coding for the two synthetic IgG binding domains (the so-called Z domains) of the pEZZ18 expression vector (Amersham Pharmacia Biotech) (9). DNA encoding the EGF-like repeat 13 of Notch (amino acids 530–560) was amplified by polymerase chain reaction from the pMtNMg plasmid (a generous gift from Dr. Artavanis-Tsakonas, Yale University, New Haven, CT; see Ref. 18), and the polymerase chain reaction product was cloned into the pT7Blue T vector (Novagen Inc., Madison, WI).

Chimeras of hEGF and Notch EGF repeat 13 were made by exchanging domains bordered by the shared cysteine residues. To generate E1N6E, which stands for hEGF containing the entire sequence of the Notch EGF repeat 13 between the first and sixth cysteine residue, the Notch EGF repeat was cleaved at the first cysteine codon with *Bsm*I and at the sixth cysteine codon with *Dra*III. This fragment was subsequently cloned into the *Bsm*I-*Sal*I site of pEZZ/FX/EGF together with a double-stranded oligonucleotide spanning the region between the *Dra*III and the *Sal*I sites. The pEZZ/FX/EGF and pEZZ/FX/E1N6E constructs were subsequently used to generate other hEGF/Notch chimeras. Exchanges at the first cysteine codon were achieved by digestion of hEGF and E1N6E with *Bsm*I, exchanges at the third cysteine codon by digestion of hEGF with NsiI, exchanges at the fourth cysteine codon by digestion of E1N6E with *Dra*I, and exchanges at the sixth cysteine codon by digestion of hEGF with *Sac*I 3′ of the sixth cysteine codon. Synthetic double-stranded oligonucleotides were used to span the region between two endonuclease restriction sites.

By digestion with *Nsi*I, the codon for the hEGF-derived methionine at position 21 (C-terminal of the third cysteine codon) was preserved, generating E3N6E/M and E3N4E/M. The M21H point mutation, as well as other, additional mutations, was introduced using the Altered SitesTM II *in vitro* mutagenesis system (Promega Inc., Madison, WI).

Expression and Purification of Recombinant Growth Factors—Wild type and mutant growth factors were expressed and secreted as ZZ/FX/growth factor fusion proteins into the periplasmic space of *Escherichia coli* KS474, a degP protease-deficient mutant (a generous gift from Drs. K. L. Strauch and J. Beckwith, Harvard University; see Ref. 22). Bacteria were grown in 2YTE (per liter: 16 g of bactotryptone, 10 g of yeast extract, 8 g of NaCl, 0.4% glucose) at 30 °C until an A_{600} of 1.5 was reached, and the periplasmic proteins were isolated as described previously (23). The fusion proteins were purified using IgG-Sepharose (Amersham Pharmacia Biotech), and the recovery was measured by an enzyme-linked immunosorbent assay based on binding competition with biotin-labeled protein A (24). The recombinant growth factors were separated from the two IgG binding domains by digestion with Factor X coupled to CNBr-activated Sepharose and further purified by an additional run on IgG-Sepharose. Dimeric forms and inactive forms with disulfide bridge mismatches were removed by reversed-phase HPLC on a Deltapak C_{18} column (Waters Associates, Milford, MA). Elution was carried out using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetate. The biological activity in the column fractions (1 ml) was tested on HER-14 cells in a binding competition assay with ^{125}I-labeled murine EGF (mEGF) (see below). Fractions containing low affinity B-loop mutants were identified by dot blot analysis using a polyclonal antibody (Ab-3) raised against recombinant wild type hEGF (Oncogene Science Inc., Cambridge, MA). Previous studies have shown that this antibody also recognizes hEGF mutants with altered B-loop sequences (11). Probed proteins were detected by a goat anti-rabbit antibody linked to horseradish peroxidase and visualized by enhanced chemiluminescence (Boehringer Mannhein).

Analysis of Mutant Fusion Proteins by SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—The presence of fusion proteins in the periplasm was verified on a 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, and the Western blots were probed with a rat anti-goat antibody linked to horseradish peroxidase to detect IgG binding domains. Enzyme activity was detected by incubation with tetramethylbenzidine/sodium dioctylsulfosuccinate/H_2O_2 in phosphate/citrate buffer (pH 5).

Quantification of Mutant (Fusion) Proteins—The amount of ZZ/FX/growth factor fusion protein present in the unpurified periplasm or in samples purified by IgG-Sepharose was estimated by measuring the total amount of IgG binding activity. This was done in a competitive enzyme-linked immunosorbent assay using protein A as a standard and biotin-labeled protein A (Sigma) as a competitor (19). The amount of growth factor obtained after the final purification by RP-HPLC was calculated from the peak area (absorption at 229 nm) using natural mEGF (Bioproducts for Science Inc., Indianapolis, IN) analyzed under the same conditions as the standard.

^{125}I-mEGF Binding Competition Assay—mEGF was iodinated using Enzymobeads (Bio-Rad) to a specific activity of ~500 Ci/mmol. HER-14 cells (NIH-3T3 cells transfected with the hEGF receptor, obtained from Dr. J. Schlessinger (New York University; see Ref. 9) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS) and grown to confluency in gelatinized 24-well dishes (1.8 cm^2). The medium was removed, and serial dilutions of wild type or mutant growth factors were added in 100 μl of DMEM containing 15 mM HEPES (pH 7.7) and 10% NCS together with 1 ng/ml ^{125}I-mEGF. After incubation for 2 h at room temperature, the cells were rinsed twice with ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin and once with ice-cold phosphate-buffered saline. Cells were then lysed by incubation with 250 μl/well 1% Triton X-100 for 1 h at room temperature. Finally, radioactivity present in the cell lysates was measured by γ counting. Experiments were performed in duplicate using natural mEGF as a standard and repeated at least twice.

Mitogenic Assays—HER-14 cells were seeded in gelatinized 24-well dishes at a density of 6.0×10^4 cells/well in 1 ml of DMEM/NCS. After 24 h of incubation, the medium was replaced by 0.9 ml of DMEM/Ham's F-12 medium (1:1) supplemented with 30 nM Na_2SeO_3, 10 μg/ml human transferrin, and 0.5% bovine serum albumin. After an additional 48 h of incubation, serial dilutions of purified growth factor were added in 0.1 ml of DMEM containing 50 mM BES (pH 6.8). Eight hours later, 0.5 μCi of [^{3}H]thymidine was added in 0.1 ml of Ham's F-12 medium. Incorporation of the tracer into cellular DNA was determined 24 h after growth factor addition. For this, cells were washed twice with phosphate-buffered saline and subsequently fixed with methanol at room temperature. After 15 min, the methanol was aspirated, and the cells were lysed in 1 ml of 0.2 N NaOH for 30 min at 37 °C as described (25). Radioactivity was determined by liquid scintillation counting. Experiments were performed in duplicate and repeated at least twice.

RESULTS AND DISCUSSION

Receptor Binding Affinity of hEGF/Notch Domain Exchange Mutants—A set of five chimeras was made initially by exchanging domains bordered by the conserved cysteine residues present in both hEGF and Notch EGF-repeat 13. They were named according to the nomenclature used in previous studies (8, 9), in which, for instance, E1N6E stands for hEGF containing Notch sequences between the first and sixth cysteine residues (Table I). All mutants were expressed and secreted into the periplasmic space of *E. coli* KS474 fused to two protein A-derived IgG binding domains. These chimeras were initially screened as fusion proteins in a binding competition assay with ^{125}I-mEGF for their ability to interact with the hEGF receptor. Their affinity was compared with that of hEGF fusion protein expressed in a parallel culture.

In the first chimera, E1N6E, the entire sequence of EGF-repeat 13 from Notch was inserted in hEGF between the first and sixth cysteine residues. E1N6E shows a high sequence homology with hEGF in the C-loop (Gly^{36}, Phe^{37} (Tyr^{37} in hEGF), Gly^{39}, and Arg^{41}) and in the region between the second and third cysteine residues (Leu^{15}, Asp^{17}, and Gly^{18}). Furthermore, both the N- and C-terminal tails of hEGF are present in E1N6E. Despite this, we were unable to detect any biological

TABLE I

Binding affinity of domain exchange mutants of hEGF and Notch

Chimeric growth factors were tested as fusion proteins with two protein A-derived IgG-binding domains, and their affinity for the hEGF receptor was compared with wild type hEGF fusion protein in a ^{125}I-mEGF binding competition assay. The amount of fusion protein was estimated in an enzyme-linked immunosorbent assay with biotin-labeled protein A. The affinities of the chimeric growth factors are expressed relative to the affinity of hEGF fusion protein: −, <0.01; ±, 0.05–0.10; +, 0.10–0.50; ++, 0.50–0.75; +++, 1.0.

The chimeric growth factors are presented schematically. Domains are exchanged bordered by the six cysteine residues C1–C6, which are present in both hEGF and Notch. hEGF sequences are presented as dashed lines, and Notch sequences are double underlined.

		Affinity
	A-loop B-loop C-loop 1 5 10 15 20 25 30 35 40 45 50	
hEGF	NSDSE C PLSHDGY C LHDGV C MYIEA..LDKYA C N C VVGYIGER C QYRDLKWWELR	
Notch	.DIDE C QSNP... C LNDGT C HDKI....NGFK C S C ALGFTGAR C QT....	
hEGF	----------	+++
Notch	C1==========C6	ND[a]
E1N6E	-----C1==========C6----	−
E3N6E	----------C3==========C6----	−
E3N4E	----------C3==========C4----------	−
E1N2E	-----C1========C2----------	±
E5N6E	----------C5==========C6----	++

[a] ND, not determined.

activity of E1N6E on HER-14 cells expressing the hEGF receptor (Table I). Instead, when only the C-loop of Notch was inserted in hEGF a chimeric protein (E5N6E) was obtained with nearly 100% binding affinity (Table I). This indicates that the Notch C-loop meets the requirements of high affinity binding, most likely because of its high sequence homology with hEGF. However, exchange of either the A-loop or the B-loop of hEGF with the corresponding sequence in Notch resulted in a chimera (E1N2E and E3N4E, respectively) with a significantly reduced binding affinity (Table I). This suggests that there are specific requirements for binding in both the A- and B-loops of hEGF that are absent in the Notch sequence.

Minimal Requirements for the A-loop for High Affinity Ligand-Receptor Interaction—NMR studies have shown that residues located around the second cysteine, which are Tyr^{13}, Leu^{15}, and His^{16} in hEGF, are in close contact with residues in the C-loop, such as Arg^{41}, forming a nonlinear binding pocket (6, 7). The latter residue, especially, has little freedom of mutation and is absolutely conserved among EGF receptor agonists (5). But also, the residues in the N-terminal domain are relatively well conserved (5), with a tyrosine or a phenylalanine at position 13; a leucine, an isoleucine, or a phenylalanine at position 15, and a histidine or an asparagine at position 16. This suggests that together with Arg^{41} and Leu^{47} in the C-terminal domain, these residues may form a nonlinear binding pocket. In EGF-repeat 13 of Notch, the third domain, located between the second and third cysteine residues, shows a very strong homology with the corresponding domain in hEGF. In contrast, the Notch A-loop sequence between the first and second cysteine shows no homology at all with any of the known EGF receptor agonists (see Table I). Most apparent are the difference in the length of the loop (4 amino acids in Notch as opposed to 7 in hEGF) and the absence of the tyrosine (or phenylalanine) just N-terminal of the second cysteine (corresponding to position 13 in hEGF).

To determine the minimal requirements of the A-loop sequence for high affinity EGF receptor binding, we introduced a tyrosine residue in E1N2E just N-terminal of the second cysteine by mutating the proline residue in the Notch sequence. This mutant was designated E1N2E/Y (Table II). Both E1N2E and E1N2E/Y were expressed and purified to homogeneity (after protein A cleavage) by RP-HPLC as described under "Experimental Procedures," and their ability to interact with the hEGF receptor was measured in a ^{125}I-mEGF binding competition assay on HER-14 cells. Fig. 1*A* shows that both mutants are able to displace radiolabeled mEGF. As expected, much higher concentrations of E1N2E than of wild type hEGF were needed to reach 50% competition, and the affinity of the chimera was calculated to be 9% of that of hEGF (in agreement with the results obtained with unpurified E1N2E fusion protein). Replacement of the proline with a tyrosine (E1N2E/Y) caused a remarkable increase in binding affinity of the hEGF/Notch chimera, resulting in an affinity close to 100%. It is concluded that for the A-loop the requirements for receptor binding are very limited in that both the size of the loop and the primary sequence may vary considerably. Only Tyr^{13} seems to be important, because its presence makes high affinity interaction with the receptor possible. For most of the residues in this region, only a few mutants have been examined in the past, showing that Pro^{7} in hEGF can be replaced by Leu or Thr and Asp^{11} can be replaced by Tyr without significant loss of binding affinity. Tyr^{13}, however, can only be replaced by a large hydrophobic residue such as Phe or Leu (reviewed in Ref. 5). The present data show, however, that even in the absence of a tyrosine at position 13, the chimeric protein can bind the receptor, albeit with a 10-fold lower affinity.

Minimal Requirements for the B-loop for High Affinity Ligand-Receptor Interaction—There is still controversy whether the B-loop is part of the binding domain and directly interacts with the EGF receptor binding pocket or whether its importance lies in the formation of a structural scaffold on which the actual site of interaction is formed. When we exchanged the B-loop of hEGF for the corresponding sequence in EGF-repeat 13 from Notch (E3N4E), a dramatic loss of receptor binding affinity was seen (Table II). This indicates that in order to bind with high affinity to the EGF receptor, there are also restrictions with respect to the B-loop sequence. One explanation for the low affinity of E3N4E might be the fact that the Notch B-loop is shorter by two amino acids. Furthermore, previous studies have shown that several residues in the B-loop may be important. At position 22, for instance, a tyrosine residue is found in hEGF, which, together with a number of other conserved aromatic residues, forms two hydrophobic clusters that are thought to be involved in maintaining the structural integrity of the polypeptide (reviewed in Ref. 5). In the Notch B-loop, this aromatic residue is replaced by aspartic acid, which has been shown to reduce the binding affinity significantly (26, 27). Furthermore, a large hydrophobic residue (Leu or Ile) may be required at position 23 (26–29) and perhaps also at position 26 (26, 27), whereas there is evidence that a small hydrophobic residue (Ala, Val, Ser, or Thr) is required at position 30 (30). In Notch, however, a lysine is found at the latter position, a

TABLE II

Binding affinity and mitogenic activity of hEGF/Notch chimeras in which hEGF-derived residues are reintroduced

Chimeric growth factors were purified to homogeneity (after protein A cleavage) by RP-HPLC, and their biological activity was compared with that of purified recombinant hEGF. The chimeric growth factors are presented schematically. Domains are exchanged bordered by the six cysteine residues C1–C6, which are present in both hEGF and Notch. hEGF sequences are presented as dashed lines, and Notch sequences are double underlined. Reintroduction of hEGF-derived amino acids into the Notch sequence is presented in capital letters. Results are the mean ± S.E. of at least three experiments.

		Affinity[a]	Mitogenicity[b]
	A-loop B-loop C-loop 1 5 10 15 20 25 30 35 40 45 50		
hEGF	NSDSE C PLSHDGY C LHDGV C MYIEA..LDKYA C N C VVGYIGER C QYRDLKWWELR		
Notch	... C QSNP... C LNDGT C HDKI....NGFK C S C ALGFTGAR C		
hEGF	----------	1	1
Notch	C1==========C6	ND[c]	ND
E1N2E	-----C1=========C2----------	0.09 ± 0.04	1.98 ± 0.93
E1N2E/Y	-----C1=======Y=C2----------	0.81 ± 0.15	1.83 ± 0.91
E3N4E	----------C3==========C4----------	0.002 ± 0.001	0.05 ± 0.02
E3N4E/MY	----------C3=MY=======C4----------	0.016 ± 0.006	0.41 ± 0.10
E3N4E/MA	----------C3=M=======A=C4----------	0.29 ± 0.17	0.93 ± 0.06
E3N4E/MYA	----------C3=MY======A=C4----------	0.87 ± 0.20	1.07 ± 0.32
E5N6E/E	----------C5======E==C6----	0.99 ± 0.31	0.81 ± 0.22
E3N6E/MYAN	----------C3=MY=======A====N==========C6----	0.34 ± 0.11	1.22 ± 0.04
E3N6E/MYANE	----------C3=MY=======A====N=========E==C6----	0.32 ± 0.10	1.13 ± 0.13
E1N6E/YMYANE	-----C1=======Y=========C3=MY=======A====N=========E==C6----	0.08 ± 0.01	0.98 ± 0.34

[a] The affinity of the hEGF/Notch chimeras for the hEGF receptor was measured in a ^{125}I-mEGF binding competition assay and is expressed as $IC_{50,hEGF}/IC_{50,mutant}$.

[b] The mitogenic activity was determined by measuring the ability to stimulate the incorporation of [^{3}H]thymidine into the cellular DNA of quiescent HER-14 cells and is expressed as $EC_{50,hEGF}/EC_{50,mutant}$.

[c] ND, Not determined.

positively charged residue with a large hydrophobic side chain. We therefore systematically reintroduced some of these residues in the B-loop of E3N4E in order to find the minimal requirements for high affinity ligand-receptor interaction.

Starting with the residues closest to the third and fourth cysteine, an alanine and a tyrosine were reintroduced at positions 30 and 22, respectively. In addition, at position 21, a methionine was reintroduced, based on our previous observation that pairwise mutations in the B-loop may have less impact on receptor binding than single point mutations (11). Four B-loop mutants were finally purified to homogeneity (after protein A cleavage) by RP-HPLC as described under "Experimental Procedures" and tested for their ability to interact with the hEGF receptor in a ^{125}I-mEGF binding competition assay on HER-14 cells. These are E3N4E, containing the entire B-loop of Notch, and the E3N4E-derived mutants E3N4E/MY, E3N4E/MA, and E3N4E/MYA (Table II and Fig. 1*B*). In agreement with the results obtained with unpurified fusion protein, E3N4E showed only marginal affinity for the hEGF receptor, just within the detection limit. Reintroduction of the methionine at position 21 was without effect (tested as a fusion protein). Introduction of the tyrosine at position 22, however, caused an 8-fold increase in binding affinity, but compared with wild type hEGF, E3N4E/MY was still 60 times less potent. Mutation of the lysine at position 30 in Notch into an alanine as in E3N4E/MA was far more effective and resulted in a more than 100-fold increase in binding affinity. When methionine 21, tyrosine 22, and alanine 30 were reintroduced into the Notch B-loop sequence of E3N4E, this was sufficient to almost completely restore the binding ability. The affinity of E3N4E/MYA was calculated to be 89% of that of hEGF.

It is concluded that for the B-loop, also, the requirements for high affinity receptor binding are limited. There seems to be a special need for a residue with a short side chain at position 30 and an aromatic residue at position 22. The latter residue lies opposite another aromatic residue at position 29 of the B-loop β-sheet of hEGF. This tyrosine residue probably also adds to the hydrophobic cluster that holds the A-loop close to the B-loop β-sheet and thus might be important for keeping the conformation of the binding domain intact. The conservative substitution of Tyr^{29} by a phenylalanine in Notch will have no effect on the binding affinity. The results with E3N4E/MYA, furthermore, show that the remaining hEGF B-loop residues (23–28) can be replaced by the corresponding Notch residues without loss of receptor binding ability. Notable is the substitution of isoleucine 23 for the positively charged lysine, because many point mutation studies have indicated that this residue can only be replaced by a large hydrophobic residue, whereas smaller and/or more hydrophilic side chains (Ala, Thr, and Asp) decrease the binding affinity considerably (5). The present data are, however, in agreement with results obtained previously in our laboratory with a hEGF/Argos chimera in which replacement of isoleucine 23 by a lysine in combination with two additional mutations in the B-loop had no effect on receptor binding affinity (11). Also, the other substitutions are quite unexpected in that they are generally not found in any of the known EGF receptor agonists, with the exception of asparagine 27, which is also present in guinea pig EGF (5). The extremely high affinity of E3N4E/MYA and E1N2E/Y is even more remarkable because in these proteins, several mutations and deletions are combined, whereas it has been shown that the effects of multiple mutations are generally cumulative (27). Most of the double mutations in the latter study, however, were performed in separate loops. Based on the present study and on results obtained previously with hEGF/Argos chimera (11), we hypothesize that within the B-loop β-sheet, single point mutations may be less well tolerated than multiple pairwise mutations. Furthermore, our data show that the B-loop can be shortened by two residues without affecting receptor binding. More extensive truncation of the B-loop, however, by four or six amino acids, resulted in a complete loss of binding affinity,[3]

[3] M. L. M. van de Poll, unpublished results.

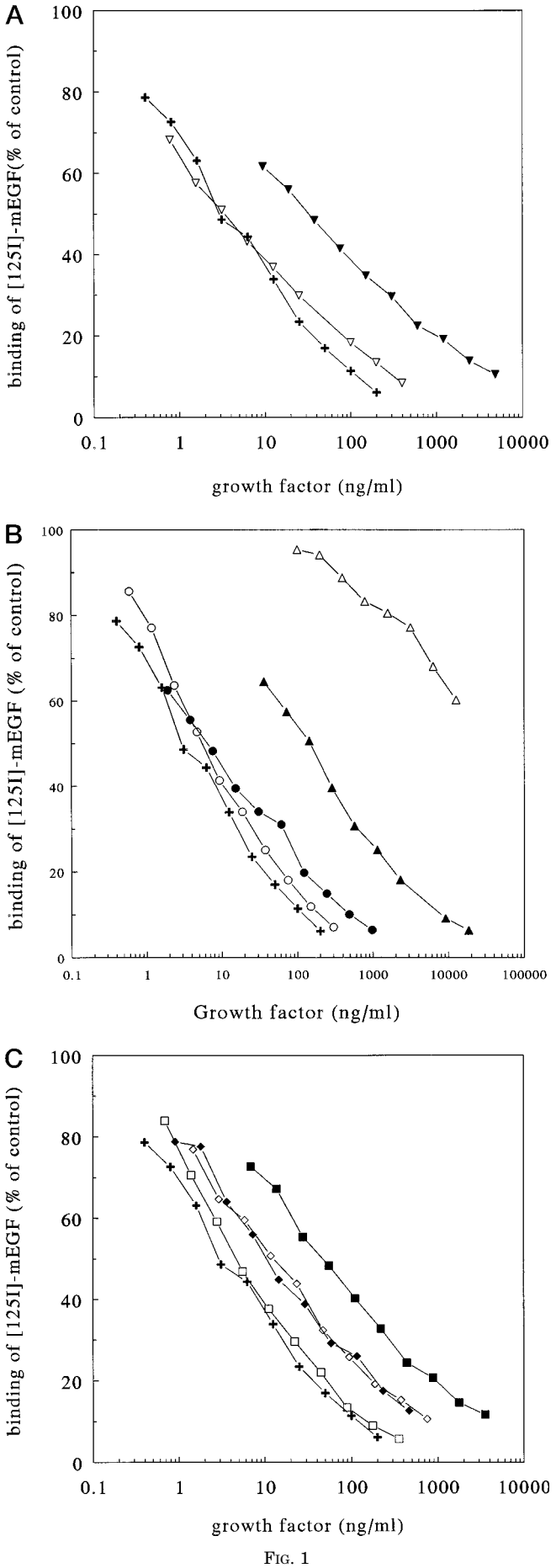

FIG. 1

and extension by as little as two residues also caused a large decrease in binding affinity (11).

The low requirements for the B-loop support the model in which the B-loop is thought to act as a scaffold. There seems to be a need for a β-sheet structure of a certain length, but further restrictions are only posed upon the residues that lie closest to the binding domain, *i.e.* the residues adjacent to the cysteines.

Requirements for the C-loop for High Affinity Ligand-Receptor Interaction—The chimeric fusion protein E5N6E, in which the hEGF C-loop was exchanged with the corresponding sequence in Notch, was able to compete with ^{125}I-mEGF for binding to the hEGF receptor. Its binding affinity was compared with hEGF fusion protein and was calculated to be 50–75% of the wild type growth factor (Table I). The relative high binding affinity of E5N6E is in agreement with the strong sequence homology of the hEGF and Notch C-loop. The amino acids at positions 36, 37, 39, and 41 are conserved or semiconserved, and at positions 34 and 35, hydrophobic residues are found in both EGF and Notch. The most apparent difference appears to be the acidic side chain of Glu^{40} in hEGF as opposed to the small hydrophobic side chain of Ala in Notch. Although other EGF receptor agonists, such as TGFα and betacellulin, also contain an alanine at position 40, replacement of Glu^{40} in hEGF by Ala has been shown to cause a 3-fold reduction in binding affinity (5). We were able to slightly increase the binding affinity of E5N6E by reintroduction of glutamic acid at position 40 (E5N6E/E). In this way, also E5N6E bound with nearly 100% affinity to the hEGF receptor (Fig. 1*C* and Table II).

Requirements for E1N6E in Order to Bind the Human EGF Receptor—In the previous sections, we focused on the requirements of individual loop sequences of hEGF and described how we were able to create hEGF/Notch chimeras with nearly 100% binding affinity for the hEGF receptor. We now sought to combine these data in experiments on E1N6E to see whether this would be sufficient to turn this unrelated EGF-like repeat into an hEGF receptor ligand. We reasoned that in addition to the essential residues for the A-, B-, and C-loops, we would also have to introduce the asparagine between the fourth and fifth cysteines. This residue is generally regarded as a hinge that determines the relative orientation of the N- and C-terminal domain and is thus crucial for the correct conformation of the binding domain (31). We then combined these mutations in E1N6E and constructed E1N6E/YMYANE. This chimeric protein was expressed and purified to homogeneity on RP-HPLC together with two additional chimeras, E3N6E/MYAN and E3N6E/MYANE, and the affinity of the mutants for the hEGF receptor was tested in a ^{125}I-mEGF binding competition assay on HER-14 cells. Fig. 1*C* and Table II show that in contrast to the parental E1N6E and E3N6E (Table I), E1N6E/YMYANE, E3N6E/MYAN, and E3N6E/MYANE were able to efficiently displace radiolabeled mEGF, showing considerable binding affinity for the hEGF receptor. Although the effect of reintroducing important hEGF-derived amino acids in the Notch sequence appeared to be less than cumulative, it was clearly sufficient to turn this Notch EGF-like repeat into a hEGF receptor agonist.

Mitogenic Activity of hEGF/Notch Chimeras—The first ob-

Fig. 1. **Binding affinity of hEGF/Notch chimeras.** The binding affinity of RP-HPLC purified chimeric proteins was measured in a ^{125}I-mEGF binding competition assay on HER-14 cells and compared with recombinant wild type hEGF. *A*, binding affinity of EGF mutants with Notch sequences in the A-loop, +, hEGF; ▼, E1N2E; ▽, E1N2E/Y. *B*, binding affinity of EGF mutants with Notch sequences in the B-loop, +, hEGF; △, E3N4E; ▲, E3N4E/MY; , E3N4E/MA; ○, E3N4E/MYA. *C*, binding affinity of hEGF (+), E5N6E (□), E3N6E/MYAN (◇), E3N6E/MYANE (◆), and E1N6E/YMYANE (■).

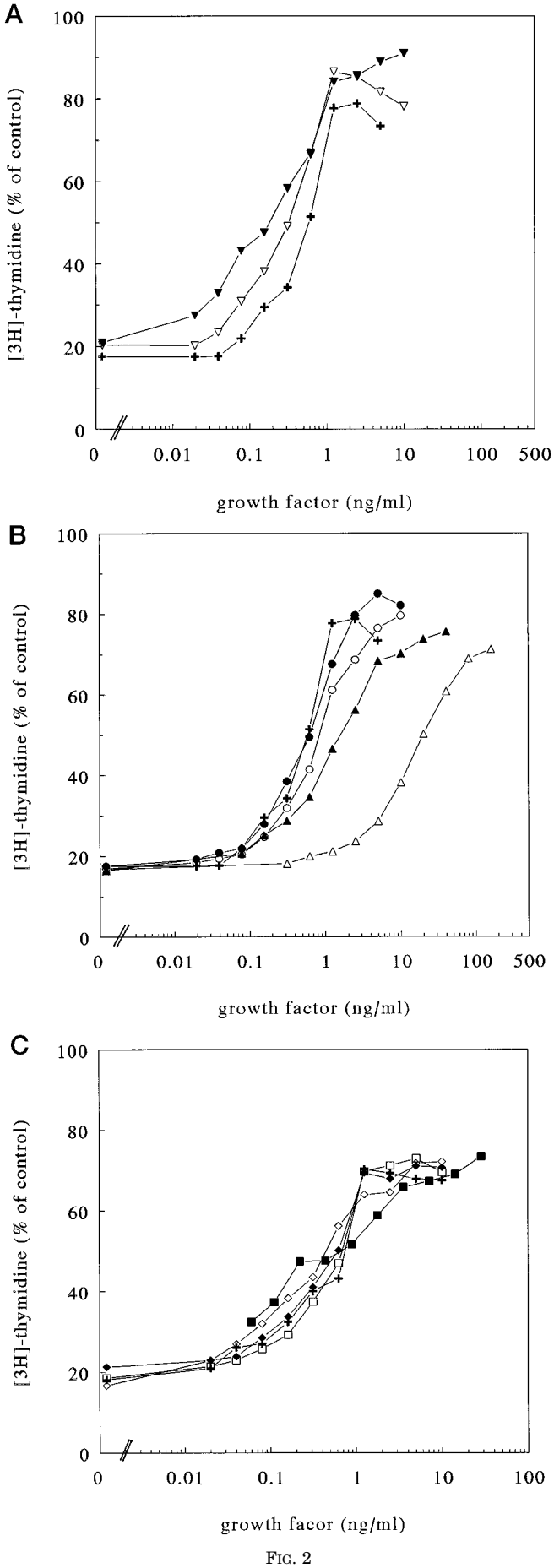

FIG. 2

jective of the present study was to gain more insight into the structure of the binding domain of hEGF receptor agonists and to determine the minimal requirements for high affinity receptor binding. The second objective was to see whether introduction of the binding domain into an unrelated biological inactive protein would result in a hEGF receptor ligand with antagonistic properties because the newly formed ligand might lack a putative activation domain.

We therefore tested the ability of the various hEGF/Notch chimeras to stimulate the incorporation of [^{3}H]thymidine into the cellular DNA of quiescent HER-14 cells and compared their mitogenic potential with wild type recombinant hEGF (Fig. 2 and Table II). The results were striking in that most of the ligands that are able to interact with the hEGF receptor are as potent as wild type hEGF in a mitogenic assay, irrespective of whether they bind with high or low affinity. Only E3N4E, which has an extremely low affinity for the hEGF receptor, is clearly less potent (by ~20-fold), but E3N4E/MY, for instance, with a calculated affinity of only 1.6%, is almost as potent as wild type hEGF.

Cellular responses are thought to be mediated by a small population of high affinity receptors (32, 33). Theoretically, the above chimeras may have a high affinity for this subset of EGF receptors and a strongly reduced affinity for the large majority of low affinity receptors. In a previous study (11), however, we have shown that even after extraction of the low affinity receptor population by Triton X-100, a 100% biologically active chimera of hEGF and Argos still had a 20-fold lower affinity than hEGF for the remaining high affinity receptors. Furthermore, in the same study, we could show, using D1 cells (IL-3-dependent myeloid cells transfected with erbB-1 only), that the relative high mitogenic potency of this hEGF/Argos chimera is also not due to the formation of more potent heterodimers of erbB-1 with other members of the erbB receptor family. Likewise, the hEGF/Notch chimeras in the present study were also as potent as wild type hEGF when tested on D1 cells (data not shown).

In conclusion, it appears that the requirements for efficient activation of the hEGF receptor are even lower than the requirements for high affinity binding. Even when the B-loop of hEGF is completely exchanged with the unrelated Notch sequence, this does not affect the bioactivity of the growth factor; also, the tyrosine at position 13 is dispensable. As mentioned above, we have previously obtained similar results with chimeras of hEGF and the *Drosophila* EGF receptor antagonist Argos (11). Also, from other research groups, more and more data accumulate showing that low affinity interaction with the receptor is often sufficient to generate a potent signal (33–35). Besides low-affinity ligands that have wild type agonistic properties, we also constructed, in the past, several high affinity ligands with superagonistic properties (10). Together with the present set of mutants, they will provide a valuable tool to study which properties of the ligand determine the final out-

Fig. 2. **Mitogenic activity of hEGF/Notch chimeras.** The ability of RP-HPLC purified chimeric proteins to stimulate the incorporation of [^{3}H]thymidine into the cellular DNA of quiescent HER-14 cells is given as a percentage of control (10% NCS). *A*, mitogenic activity of EGF mutants with Notch sequences in the A-loop. +, hEGF; ▼, E1N2E; ▽, E1N2E/Y. [^{3}H]Thymidine incorporation in the presence of 10% NCS was 79,500 ± 2500 cpm; without growth factor addition, it was 15,200 ± 1400 cpm. *B*, mitogenic activity of EGF mutants with Notch sequences in the B-loop. +, hEGF; △, E3N4E; ▲, E3N4E/MY; , E3N4E/MA; ○, E3N4E/MYA. [^{3}H]Thymidine incorporation in the presence of 10% NCS was 77,800 ± 3000 cpm; without growth factor addition, it was 13,200 ± 350 cpm. *C*, mitogenic activity of hEGF (+), E5N6E (□), E3N6E/MYAN (◇), E3N6E/MYANE (◆), E1N6E/YMYANE (■). [^{3}H]Thymidine incorporation in the presence of 10% NCS was 66,100 ± 2100 cpm; without growth factor addition, it was 12500 ± 1100 cpm.

come in terms of mitogenic response. So far, high mitogenic potency has been found not to be due to the formation of more potent erbB heterodimers because a similar high mitogenic activity was seen on D1 cells that express only erbB1 (10, 11). Further research will be necessary to see whether the two phenomena described above are related and can be explained by one and the same model.

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