Identification of the Minimal Requirements for Binding to the Human Epidermal Growth Factor (EGF) Receptor Using Chimera 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.

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 Arg31 and Leu47, 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 Arg31 and Gln33 are in close contact with Tyr13, Leu15, and His16, 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 ARG45 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. 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).
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. Arg11) 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 Biotechnol. Oxford, United Kingdom) was linked to the 5’ end to the sequence for the recognition sequence (Ile-Glu-Gly-Arg) of the proteolytic enzyme factor X (21). The resulting BamHI-SalI 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 pEZZ/18 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 pMMN1 plasmid (a generous gift from Dr. Aris-Tsakonas, Yale University, New Haven, CT; see Ref. 18), and the polymerase chain reaction product was cloned into the pTZBlue 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 BsmI and at the sixth cysteine codon with DraI. This fragment was subsequently cloned into the BsmI-SalI site of pEZZ/FX/EGF together with a double-stranded oligonucleotide spanning the region between the DraI and SalI 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 BsmI and E1N6E with BsnI, exchanges at the third cysteine codon by digestion of hEGF with NsiI, exchanges at the fourth cysteine codon by digestion of E1N6E with DraI, and exchanges at the sixth cysteine codon by digestion of hEGF with SalI 3′ of the sixth cysteine codon. Synthetic double-stranded oligonucleotides were used to span the region of the gene. The cDNA for E1N6E 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 pTZBlue T vector. The recombinant growth factors were purified using IgG-Sepharose and further purified by an additional enzymelinked 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.

**M1-EGF 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 60-mm dishes (1.8 cm²). The medium was removed and growth factor addition. For this, cells were washed twice with phosphate-buffered saline, fixed with methanol at room temperature, and radioactivity present in the sample was detected by exposure to X-ray film. The amount of mEGF bound 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.

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**Expression and Purification of Recombinant Growth Factors—**Wild type and mutant growth factors were expressed and secreted as ZZ/FX/A Deltapak C18 column (Waters Associates, Milford, MA). Elution was done with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. The resulting 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 60-mm dishes (1.8 cm²). The medium was removed and growth factor addition. For this, cells were washed twice with phosphate-buffered saline, fixed with methanol at room temperature, and radioactivity present in the sample was detected by exposure to X-ray film. The amount of mEGF bound 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.
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 Tyr13, Leu15, and His16 in hEGF, are in close contact with residues in the C-loop, such as Arg11, 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 16. This suggests that there are specific requirements for binding in both the A- and B-loops of hEGF for high affinity.

**Minimal Requirements for the A-loop for High Affinity Ligand-Receptor Interaction**—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 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 125I-mEGF binding competition assay on HER-14 cells. Fig. 1A 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 Tyr13 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 Pro7 in hEGF can be replaced by Leu or Thr and Asp11 can be replaced by Tyr without significant loss of binding affinity. Tyr13, 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.

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TABLE II

|                | Affinity | Mitogenicity |
|----------------|----------|--------------|
|                | A-loop | B-loop | C-loop |                | 1 | 1 |
| hEGF           | NSDSE   | C       | 1       | N       | 0.09 ± 0.04 | 19.8 ± 0.93 |
| Notch          | QSNP    | C       | 1       | N       | 0.81 ± 0.15 | 18.3 ± 0.91 |
| hEGF           | C       | 1       | E       | C       | 0.002 ± 0.001 | 0.05 ± 0.02 |
| Notch          | C       | 1       | E       | C       | 0.016 ± 0.006 | 0.41 ± 0.10 |
| hEGF           | C       | 1       | E       | C       | 0.29 ± 0.17 | 0.93 ± 0.06 |
| Notch          | C       | 1       | E       | C       | 0.87 ± 0.20 | 1.07 ± 0.32 |
| E5N6E/MYA      | C       | 1       | E       | C       | 0.99 ± 0.31 | 0.81 ± 0.22 |
| hEGF           |        | C       | 1       | E       | 0.34 ± 0.11 | 1.22 ± 0.04 |
| Notch          |        | C       | 1       | E       | 0.32 ± 0.10 | 1.13 ± 0.13 |
| E5N6E/MYAN     | C       | 1       | E       | C       | 0.08 ± 0.01 | 0.98 ± 0.34 |
| hEGF           |        | C       | 1       | E       | 0.01 ± 0.001 | 0.05 ± 0.02 |
| Notch          |        | C       | 1       | E       | 0.016 ± 0.006 | 0.41 ± 0.10 |
| hEGF           |        | C       | 1       | E       | 0.29 ± 0.17 | 0.93 ± 0.06 |
| Notch          |        | C       | 1       | E       | 0.87 ± 0.20 | 1.07 ± 0.32 |
| E5N6E/MYA      |        | C       | 1       | E       | 0.99 ± 0.31 | 0.81 ± 0.22 |
| hEGF           |        | C       | 1       | E       | 0.34 ± 0.11 | 1.22 ± 0.04 |
| Notch          |        | C       | 1       | E       | 0.32 ± 0.10 | 1.13 ± 0.13 |
| E5N6E/MYAN     |        | C       | 1       | E       | 0.08 ± 0.01 | 0.98 ± 0.34 |

a The affinity of the hEGF/Notch chimeras for the hEGF receptor was measured in a 125I-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 [3H]thymidine into the cellular DNA of quiescent HER-14 cells and is expressed as EC_{50, hEGF}/EC_{50, mutant}.

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 125I-mEGF binding competition assay on HER-14 cells. These are E3N4E, containing the entire B-loop of Notch, and the E3N4E-derived mutants E3N4E/MA, E3N4E/MA, and E3N4E/MYA (Table II and Fig. 1B). In agreement with the results obtained with unpurified protein, E3N4E showed only marginal affinity for the hEGF receptor, just within the detection limit. Introduction of the tyrosine at position 22, however, caused an 8-fold increase in binding affinity, but compared with wild type hEGF, E3N4E/MYA 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 Tyr29 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.
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 [125I]-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 Glu40 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 Glu40 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. 1C 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 125I-mEGF binding competition assay on HER-14 cells. Fig. 1C 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-
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 [3H]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-
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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