Non-linear antigenic regions in epidermal growth factor (EGF) and transforming growth factor α (TGF α) studied by EGF–TGF α chimaeras

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With the help of 16 chimaeras between human epidermal growth factor (hEGF) and human transforming growth factor α (hTGF α), a detailed analysis was performed on the epitope recognized by two polyclonal antibodies raised against hEGF, and one polyclonal antibody raised against hTGF α . All three antibodies recognized essentially the same antigenic site, a non-linear and conformation-dependent sequence that is located near the second and fourth disulphide-bonded cysteines and that includes the start of the B-loop β -sheet. The epitope recognized by the anti-hEGF antibodies was further characterized using 8

chimaeras between hEGF and an EGF-repeat from *Drosophila* Notch and was found to include Met²¹, Ala³⁰ and Asn³². All three polyclonal antibodies were able to neutralize the biological activity of the respective growth factor when tested on 32D murine haematopoietic progenitor cells transfected with ErbB-1, indicating that the receptor binding domain is shielded upon binding of the antibody.

Key words: antibodies, conformation-dependent, epitope mapping, discontinuous.

INTRODUCTION

Human epidermal growth factor (hEGF) and human transforming growth factor α (hTGF α) are single-chain polypeptides of 53 and 50 amino acids respectively [1]. The polypeptide chain is folded in a well-defined conformation, which is largely determined by the presence of six identically spaced disulphide-bonded cysteine residues and two highly conserved glycine residues. The structures of hEGF and hTGF α can be divided into three disulphide-bonded loops: the A-loop (residues 6–19) containing some α -helical structure, the B-loop (residues 20–31) forming a two-stranded anti-parallel β -sheet, and the C-loop (residues 33–42), which is part of a second β -sheet structure. Between the fourth and fifth cysteine residues a single amino acid (Asn³² in hEGF and Val³³ in hTGF α) functions as a hinge. The loops are linked to linear sequences at both the N- and C-termini [1].

EGF and TGF α bind to and activate a tyrosine kinase receptor known as the EGF receptor or ErbB-1, and by virtue of this interaction they are potent mitogens that stimulate cellular proliferation and differentiation in a wide variety of cell types [2,3]. High expression of members of the EGF family, in particular TGF α , has been found in several human cancers and the co-expression of EGF receptors in these tumours increases the possibility of autocrine growth stimulation. In general, high expression of TGF α in human malignant effusions has been found to correlate with poor prognosis [4–6]. In the past, antisera have been generated as a diagnostic tool to detect EGF and TGF α expression in tissue samples. In addition, monoclonal and site-specific polyclonal antibodies have been used in fundamental research to identify functional epitopes.

Antigenic regions in TGF α have been identified in the past either by the ability of anti-peptide antisera to recognize native TGF α [7–9] or by the ability of anti-TGF α antibodies to react with linear peptide fragments that cover the entire sequence of

the protein [10–12]. In this way both the N-terminal tail (residues 1–9), the B-loop (residues 22–34) as well as the C-terminus (residues 34–50) were found to be immunoreactive sites.

Similar studies on the antigenic sites in EGF have recognized the B-loop (residues 22–32) as an immunodominant region [13]. On the other hand, when polyclonal antisera were raised against the intact protein all kinds of monospecific antibodies could be identified, by affinity purification, against peptide fragments encompassing the entire EGF sequence [13]. This indicates that antigenic regions are present over the entire molecule. However, it gives no information on the most prevalent immunoreactive site in EGF.

Using linear peptide fragments to either map epitopes or identify immunodominant regions on proteins has the disadvantage that only linear, non-conformational, epitopes can be detected, whereas discontinuous or conformational epitopes remain unrevealed. Thus several monoclonal antibodies raised against native EGF or TGF α were found to be unable to bind any of the linear peptide fragments and showed no affinity for the intact protein after reduction of disulphide bridges. This indicates that they recognize a thus far unknown conformational epitope [12,13].

To identify regions important for receptor binding, we have previously constructed a large panel of hEGF–hTGF α chimaeras [14,15] as well as chimaeras between hEGF and an EGF repeat from *Drosophila* Notch, a 300 kDa transmembrane protein with no apparent growth-factor activity [16,17]. In these chimaeras domains have been exchanged at the sites of the highly conserved cysteine residues to ensure optimal protein folding and biological activity. Fine-tuning to individual amino acids was achieved by selective reintroduction of EGF-derived residues [16]. With these chimaeras we were able to identify the minimal requirements for EGF receptor recognition in terms of loop-size and amino acid identity. Several antibodies raised against native EGF or TGF α are commercially available nowadays, but the epitopes they

Abbreviations used: hEGF, human epidermal growth factor; hTGF α , human transforming growth factor α ; anti-hTGF α 9129, residues 9129–8024 of mature human recombinant TGF α ; IGF-1, type I insulin-like growth factor; IL-3, interleukin-3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; ECL, enhanced chemiluminescence.

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recognize are unknown. Since anti-EGF and anti-TGF α antibodies are usually not cross-reactive, due to the relatively low sequence similarity (42 %) between these related growth factors, EGF-TGF α chimaeras can be expected to be valuable tools for epitope mapping that will overcome the limitations encountered by using linear peptide sequences. In the present study we set out to identify the most predominant antigenic site(s), including non-linear or conformational epitopes, recognized by three commercially available polyclonal antisera, two of which were raised against native hEGF and one against native hTGF α .

EXPERIMENTAL

Antibodies

Ab-3 (Oncogene Research Products, Cambridge, MA, U.S.A.) and BT-580 (Biomedical Technologies Inc., Stoughton, MA, U.S.A.) are rabbit polyclonal antibodies raised against recombinant hEGF. They are specific for hEGF and show no cross-reactivity with hTGF α . A sheep polyclonal antibody directed against residues 9129–8024 of mature human recombinant TGF α (anti-hTGF α 9129) was purchased from Biogenesis Ltd. (Poole, Dorset, U.K.). It recognizes TGF α from human, rat and bovine origin, but does not react with EGF. The monoclonal antibody 8B-2 was generously supplied by NV Organon (Oss, The Netherlands). It was raised against a synthetic circular peptide corresponding to residues 34–50 of hTGF α .

Chimaeric growth factors

EGF-TGF α chimaeras or chimaeras between hEGF and an EGF-like repeat (amino acid residues 530–560) from *Drosophila* Notch [17] were constructed by exchanging domains bordered by the highly conserved cysteine residues, as previously described by us [15,16]. The constructs were linked at the 5' end to the sequence coding for the recognition sequence (Ile-Glu-Gly-Arg) of the proteolytic enzyme Factor X, and cloned into the *Bam*HI-*Sa*I site of the pEZZ18 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden), 3' of the sequence encoding two synthetic IgG-binding domains (Z-domains).

Wild-type and mutant growth factors were expressed and secreted as ZZ-Factor X-growth-factor fusion proteins into the periplasmic space of *Escherichia coli* KS474 and purified as described previously [15]. The isolated fusion proteins were digested with Factor X, and an additional run on IgG-Sepharose was performed to remove the two IgG-binding domains. Finally, active growth factors were separated from inactive dimers and other disulphide mismatches by reverse-phase HPLC.

Dot-blot procedure

Nitrocellulose (Protran BA 79; 0.1 μ m pore size; Schleicher and Schuell, Dassel, Germany) was pre-wetted in 25 mM Tris/HCl (pH 7.3)/192 mM glycine/20 % (v/v) methanol. Recombinant wild-type and mutant growth factors (50 ng) were dissolved in 50 μ l of 1 M acetic acid and spotted on to the nitrocellulose using dot-blot apparatus. The blot was subsequently dried and incubated for 30 min in 20 mM Tris/HCl (pH 7.3)/150 mM NaCl/0.5 % (v/v) Tween-20 (TBS-T) containing 0.1 % (w/v) ovalbumin to block non-specific binding sites. Incubation with specific primary antibodies took place for 16 h at 4 °C using a 1:1000 (polyclonal antibodies) or 1:500 (monoclonal antibody) dilution in TBS-T/0.1 % ovalbumin. Probed proteins were detected by incubation for 2 h at room temperature with a secondary antibody linked to horseradish peroxidase (Nordic Immunological Lab., Tilburg, The Netherlands) at a 1:25000 dilution in TBS-T/0.1 % ovalbumin. For visualization, enhanced

chemiluminescence (ECL; Boehringer Mannheim, Mannheim, Germany) was used.

Cell proliferation assay

32D murine haematopoietic progenitor cells, transfected with ErbB-1 [18], were grown in RPMI 1640 medium supplemented with antibiotics, 10 % (v/v) heat-inactivated fetal-calf serum and 0.5 ng/ml interleukin-3 (IL-3) [19]. Prior to stimulation, the cells were washed free of IL-3 and resuspended in RPMI 1640 medium at a concentration of 5×10^5 cells/well. Serial dilutions of polyclonal antibodies were added, together with 2.5 ng/ml hEGF or hTGF α . After 20 h at 37 °C, cell survival was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [18]. For this, cells were incubated for an additional 2 h at 37 °C with MTT (0.1 mg/ml) and the formation of dark-blue formazan crystals by living cells was quantified, after lysis of the cells with acidic propan-2-ol, by reading the absorbance at 540–630 nm.

RESULTS

Chimaeric proteins

In Figure 1 a schematic presentation is given of two sets of chimaeric proteins containing hEGF sequences; EGF-TGF α chimaeras [14,15] and EGF-Notch chimaeras [16]. The EGF-TGF α chimaeras are named according to the nomenclature introduced previously [14], in which, for instance, T3E stands for a ligand with TGF α sequences N-terminal and EGF sequences C-terminal of the third cysteine residue. A similar nomenclature is used for domain exchange mutants of hEGF and an EGF-repeat from Notch [16]. In cases where hEGF-derived amino acids were reintroduced into the Notch sequence this is indicated by capitals. Thus E1N2E stands for EGF in which the entire sequence between the first and second cysteine residue has been replaced by the corresponding sequence in Notch and E1N2E/Y stands for the same mutant in which the hEGF-derived tyrosine residue (position 13 in hEGF) has been reintroduced into the Notch sequence (Figure 1).

Mapping of the epitope recognized by polyclonal antibodies raised against native hEGF

The wild-type recombinant growth factors hEGF and hTGF α , as well as 16 EGF-TGF α chimaeras, were spotted on to a nitrocellulose filter and incubated with either Ab-3 or BT-580 (both polyclonal antibodies were raised against native recombinant hEGF). Probed proteins were detected by a goat anti-rabbit antibody linked to horseradish peroxidase and were visualized by enhanced chemiluminescence. Films were exposed for different time intervals ranging from 5–120 s in order to detect small differences in the efficiency of recognizing the various epitopes presented by the chimaeras. Table 1 gives a semi-quantitative analysis of 5 (Ab-3) and 2 (BT-580) independent experiments. Representative examples are shown in Figure 2. The antibodies show no cross-reactivity with hTGF α or murine EGF, as indicated by the fact that the signal remains below the background (0.1 % BSA), even after long exposure times.

Chimaeras with hTGF α sequences N-terminal of the third cysteine residue (T1E, T3E and T3E4T) were well recognized by Ab-3 (Figure 2, top panel), although T3E generally needed longer exposure to give the same signal as native hEGF. Also the presence of hTGF α sequences C-terminal of the fourth cysteine residue (E4T, E5T, E6T, T3E4T, E4T6E, E6ET and E6TE) did not affect the ability of Ab-3 to recognize the protein. All these chimaeras contain hEGF sequences between the third and fourth

Figure 1 Sequence alignment of hEGF, hTGF α and *Drosophila* Notch EGF-like repeat 13, and schematic presentation of chimaeric growth factors

Domains exchanged are bordered by the six cysteine residues (C1–C6), which are present in hEGF, hTGF α and Notch. hEGF sequences are presented as dashed lines and hTGF α sequences as asterisks. In the EGF/Notch chimaeras the Notch sequence that is introduced in hEGF is given in capitals. In cases where hEGF-derived amino acids are reintroduced into the Notch sequence these are underlined.

cysteine residues, which indicates that the B-loop contains the main immunoreactive site. Indeed, all chimaeras that contained hTGF α sequences in this region (E3T, T4E, T6E, T4E6T, T6ET and T6TE) were not recognized by Ab-3. A notable exception, however, was E3T4E. The finding that Ab-3 reacted with both T3E4T and E3T4E could be due to the polyclonal nature of the antibody; however, when the B-loop of hEGF was replaced by the corresponding sequence of Notch EGF-repeat 13 instead of hTGF α (E3N4E), Ab-3 was unable to bind to the chimaeric protein. The difference in reactivity between E3T4E and E3N4E might be due to the higher sequence similarity between the B-loops of hEGF and hTGF α compared with that of Notch, but it also suggests that only part of the B-loop is required to bind the antibody. To investigate this, variants of E3N4E were tested in which hEGF-derived amino acids were reintroduced. Figure 2 (top panel) shows that the presence of alanine at position 30 in combination with methionine at position 21 (E3N4E/MA) especially increased the reactivity of the chimaeric protein with Ab-3. Although these two residues in the B-loop appeared to be sufficient for antibody recognition, it did not explain why E3T4E

having an arginine at position 21 was recognized and other chimaeras with the same B-loop such as E3T and T4E were not. Therefore residues outside of the B-loop must also add to the epitope, and apparently these flanking regions must be present in order to recognize chimaeras with a hTGF α B-loop. In contrast with E3T, the EGF–Notch chimaera E3N6E/MYAN was well recognized. In this protein, C-terminal of the B-loop, only the asparagine at position 32 and the C-terminal tail were EGF-derived which indicated that either separately or together these residues may add to antibody recognition.

Using another polyclonal antibody (BT-580) raised against native hEGF, similar results were obtained (Table 1 and Figure 2, bottom panel). Similarly to Ab-3 this antibody recognized the chimaeras E4T, E5T, E6T, T3E4T, E4T6E, E6ET, E6TE and T1E that all have a hEGF B-loop. In addition E3T4E and the hEGF–Notch chimaeras E3N4E/MA and E3N6E/MYAN were again well recognized, whereas other chimaeras having hTGF α or Notch sequences in the B-loop were not. Small differences in the epitopes recognized by Ab-3 and BT-580 were, however, observed. Whereas T3E was slightly less recognized than T3E4T

Table 1 Semiquantitative analysis of the ability of antibodies raised against native hEGF (Ab-3 and BT-580), native hTGF α (9129) or hTGF α residues 34–50 (8B-2) to recognize EGF–TGF α chimaeras or EGF–Notch chimaeras

++, well recognized; +, moderately recognized; $\pm$, slightly recognized; –, not recognized; ND, not determined.

	Anti-EGF Ab-3 (n = 5)	Anti-EGF BT-580 (n = 2)	Anti-TGF α 9129 (n = 3)	Anti-TGF α 8B-2 (n = 2)
hEGF	++	++	–	–
hTGF α	–	–	++	++
murine EGF	–	ND	ND	ND
T1E	++	+	–	ND
T3E	+	–	–	–
T4E	–	–	++	–
T6E	–	–	++	–
E3T	–	–	–	+
E4T	++	+	–	++
E5T	++	+	–	++
E6T	++	+	–	+
T3E4T	++	++	++	++
E3T4E	++	+	–	–
T4E6T	–	–	$\pm$	ND
E4T6E	++	++	–	ND
T6ET	$\pm$	–	++	ND
T6TE	–	–	++	ND
E6ET	++	++	–	ND
E6TE	++	++	–	ND
E3N4E	–	$\pm$	ND	ND
E3N4E/MY	+	$\pm$	ND	ND
E3N4E/MA	++	++	ND	ND
E3N4E/MYA	+	$\pm$	ND	ND
E3N6E/MYAN	+	++	ND	ND
E5N6E/E	++	ND	ND	ND
E1N2E	++	ND	ND	ND
E1N2E/Y	++	ND	ND	ND

by Ab-3, it was not recognized at all by BT-580. In the native protein the peptide chain is folded in such a way that residues in the A-loop are in close contact to residues in the C-loop [20]. Although T3E was found to be as biologically active as hEGF and hTGF α [14], the specific combination of a hTGF α A-loop and a hEGF C-loop in T3E may have caused a slight change in the conformation of the epitope recognized by these antibodies, and the inability of BT-580 to react with T3E therefore suggests that this antibody is more sensitive to conformational changes. Furthermore, the finding that E4T6E seemed to react better with the antibody than E4T, while E6ET and E6TE (see Figure 1 for definition) also seemed to react better than E6T, suggests that the linear C-terminal tail also adds to recognition of the protein by BT-580.

The above results indicate that both polyclonal antibodies directed against native hEGF recognize essentially the same discontinuous conformational epitope. In agreement with this, reduction of hEGF by β -mercaptoethanol appeared to disrupt the epitope, causing a strong decrease in the ability of the antibodies to bind to the protein (results not shown).

Mapping of the epitope recognized by a polyclonal antibody raised against native hTGF α

The same set of hEGF–hTGF α chimaeras was used to map the epitope recognized by anti-hTGF α 9129. In Table 1 a semi-quantitative analysis of three independent experiments is given, and Figure 3 shows a representative dot-blot result. The antibody was unable to recognize chimaeras that had EGF sequences N-

Figure 2 Representative dot-blot analysis of the ability of the anti-hEGF polyclonal antibody Ab-3 (top panel) and BT-580 (bottom panel) to recognize EGF–TGF α and EGF–Notch chimaeras

Each protein (50 ng) was spotted on to nitrocellulose and the antibody was tested at a dilution of 1:1000. The reactivity of Ab-3 was tested 5 times and the reactivity of BT-580 was tested 2 times. Visualization was by ECL and the film was exposed for 15 s. mEGF, murine EGF.

terminal of the third cysteine residue (E3T, E4T, E5T, E6T, E3T4E and E4T6E), which indicates that this region contains an important immunoreactive site. On the other hand, even when the entire sequence N-terminal of the third cysteine residue was TGF α (T3E), this was clearly not sufficient for antibody recognition. Therefore either the conformation of the epitope was changed due to the specific combination of EGF and TGF α sequences, or additional regions in TGF α needed to be present in order for the antibody to bind to the protein. These could be either residues in the B-loop or residues C-terminal of the fourth cysteine residue, since both T4E and T3E4T were well recognized.

Although fine-tuning to individual amino acids was not possible with the available chimaeras, the above results suggest that, as for the anti-EGF antibodies, the epitope recognized by anti-hTGF α 9129 is a conformational non-linear epitope that contains residues lying at the start of the main B-loop β -sheet.

Figure 3 Representative dot-blot analysis of the ability of anti-hTGF α 9129 to recognize EGF-TGF α chimaeras

Each protein (50 ng) was spotted on to nitrocellulose and the antibody was tested at a dilution of 1:1000. The experiment was repeated twice. Visualization was by ECL, and the film was exposed for 20 s.

Figure 4 Representative dot-blot analysis of the ability of the monoclonal antibody 8B-2 raised against a synthetic circular peptide corresponding to residues 34-50 of hTGF α

Each protein (50 ng) was spotted on to nitrocellulose and the antibody was tested at a dilution of 1:500. The experiment was repeated once. Visualization was by ECL, and the film was exposed for 10 s.

Mapping of the epitope recognized by a monoclonal antibody raised against hTGF α residues 34-50

In order to validate the results obtained above with EGF-TGF α chimaeras, we performed a control experiment with 8B-2, a

Figure 5 Inhibition of growth factor-induced cell survival by polyclonal antibodies directed against native hEGF (Ab-3 and BT-580) or hTGF α (9129)

D1 cells expressing ErbB-1 were deprived of IL-3 and subsequently incubated with 2.5 ng/ml hEGF (top panel) or hTGF α (bottom panel), together with serial dilutions of the polyclonal antibodies. Cell survival was measured after 24 h using an MTT assay, as described in the Experimental section.

monoclonal antibody raised against a synthetic circular peptide corresponding to residues 34-50 of hTGF α . In spite of the fact that the peptide used as an immunogen was not linear, the antibody was found to recognize hTGF α better when the growth factor was reduced by β -mercaptoethanol than when the growth factor was its native conformation (results not shown). Therefore the chimaeric proteins were also tested under reducing conditions. As expected, 8B-2 was able to bind E3T, E4T, E5T and T3E4T, which are all chimaeras that contain TGF α residues 34-50 (Table 1 and Figure 4). In agreement with previous results obtained with an antiserum raised against the same peptide sequence [10], 8B-2 could also recognize chimaeras that contained only TGF α residues 43-50 (E6T). All other chimaeras tested were negative.

Inhibition of growth-factor-induced cell survival by polyclonal antibodies directed against hEGF or hTGF α

IL3-dependent 32D haematopoietic cells transfected with ErbB-1 (D1 cells) can survive in the absence of IL-3 through activation of ErbB-1 by one of its ligands. To investigate the ability of the above polyclonal antibodies to neutralize the biological activity of hEGF or hTGF α , D1 cells were deprived of IL-3 and subsequently incubated with the growth factors at a concentration of 2.5 ng/ml, which gave maximum growth stimulation (results not shown). Serial dilutions of the antibodies were added at the same time and their effect on growth-factor-dependent cell survival was measured in an MTT assay, as described in the Experimental section. Figure 5 shows that all three polyclonal antibodies inhibited the growth-factor-induced proliferation of D1 cells in a dose-dependent and growth-factor-specific way. This suggests that these antibodies recognize an epitope that is either part of or lies close to the binding interface.

DISCUSSION

Using synthetic peptide fragments several immunoreactive sites in EGF and TGF α have previously been identified, including the N-terminus, the B-loop β -sheet and the C-terminus [7–13]. A major disadvantage of the approach used, however, was that only linear non-conformational epitopes were identified. Discontinuous conformational epitopes may have remained undetected. Thus in previous studies several monoclonal antibodies raised against the native growth factors were unable to react with synthetic linear peptides, and the epitopes which they recognized remained unknown [12,13]. A more detailed analysis of antigen–antibody interaction can be obtained by the use of random peptide libraries, either displayed on phage or chemically synthesized [21,22]. Such peptide libraries have been used successfully to fine-tune linear epitopes at the amino acid level [23–26]. Furthermore, the large sequence diversity created in this way may result in the selection of peptides that mimic discontinuous binding sites. Although a few examples are known in which so-called ‘mimotopes’ were found [25,27–30], most attempts to identify discontinuous epitopes in this way have failed.

In the present study we have shown that chimaeras of hEGF and hTGF α can be used successfully to identify non-linear conformational epitopes. The most antigenic site on hEGF, which was recognized by two polyclonal antibodies raised against the native protein, was found to be discontinuous and to contain residues close to the third cysteine residue (including Met²¹ at the start of the B-loop β -sheet) and near the fourth cysteine residue (including Ala³⁰ at the end of the B-loop β -sheet and the hinge residue Asn³²). Analysis of the epitope recognized by anti-TGF α was less detailed, but the data obtained in the present study also suggest that in TGF α the same region is immunoreactive. Although we cannot rule out the possibility that only some of the residues implicated in this study are in direct contact with the antibody and that others serve for efficient and correct presentation of the epitope, the fact that the antibodies only recognize the proteins when non-reduced (results not shown) indicates that the epitope is at least conformation-dependent. Furthermore, since two-dimensional NMR studies have shown that in the three-dimensional structure the above residues are in close contact and that Met²¹ forms a hydrogen bond with Ala³⁰ in the double-stranded anti-parallel β -sheet [20] (Figure 6), this makes it likely that together these residues form the reactive site.

All three polyclonal antisera were able to block the biological activity of the respective growth factor in a proliferation assay on

Figure 6 (Upper panel) schematic presentation of hEGF showing the B-loop β -sheet with the antigenic site and (lower panel) a space-filling representation of the solvent accessible structure of hEGF

The residues that form the antigenic site are shown in colour and are found to be clustered on the outside of the molecule. An animated version of Figure 6 can be accessed via the URL: <http://www.BiochemJ.org/bj/349/bj3490267add.htm>. Met²¹ is located at the start of the B-loop β -sheet near the third cysteine residue and opposite to Ala³⁰, which is located at the end of the B-loop. Together with the hinge residue, Asn³², they form an important discontinuous epitope recognized by polyclonal antibodies raised against native hEGF. The residues indicated in grey may also add to the antigenic site.

D1 cells expressing ErbB-1, which indicates that the receptor binding domain is shielded upon binding of the antibody [20]. Increasing evidence exists that the binding domains of hEGF and

hTGF α for ErbB-1 are non-linear and consist of residues from the A-loop, the C-loop and the C-terminal tail [16,20,31–33]. Furthermore, we have previously found that Met²¹ and Ala³⁰ in the B-loop and the hinge residue, Asn³², are also essential for high-affinity ligand–receptor interaction [16], probably because they guarantee the correct conformation of the binding domain. The epitope recognized in the present study clearly overlaps with the above region.

Chimaeras between related proteins that show no cross-reactivity have been successfully used in the past to map epitopes recognized by specific monoclonal antibodies [34–39]. For example, chimaeras between the human and the chicken EGF receptor have been used to map domains recognized by various monoclonal antibodies raised against the human EGF receptor [34], and chimaeras between the human type I insulin-like growth factor (IGF-1) receptor and the human insulin receptor were used to map the epitope of IGF-1-receptor-specific monoclonal antibodies to different regions of the extracellular domain [35]. More recently four chimaeras have been constructed between human E- and L-selectins through which the epitope recognized by a neutralizing antibody, directed against human E-selectin, was assigned to the lectin domain [36]. In the above studies only a limited number of chimaeras were available and relatively large domains were exchanged. Fine-tuning was eventually achieved by introducing random amino acid substitutions.

In the present study, with the help of 16 EGF–TGF α chimaeras and 8 EGF–Notch chimaeras a detailed analysis was performed on the epitopes recognized by polyclonal antibodies directed against native hEGF and hTGF α . A thus far unknown major antigenic site was identified as a non-linear and conformation-dependent region involved in receptor binding.

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