The linear C-terminal regions of epidermal growth factor (EGF) and transforming growth factor- α bind to different epitopes on the human EGF receptor

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Epidermal growth factor (EGF) and transforming growth factor- α (TGF α) bind with similar affinities in a competitive fashion to the human EGF receptor, and basically induce similar mitogenic responses. In spite of the fact that EGF and TGF α are structurally alike, it is still not clear if the two growth factors bind the receptor in an identical manner. The observation that the 13A9 antibody blocks binding of TGF α , but not that of EGF, to the human EGF receptor [Winkler, O'Connor, Winget and Fendly (1989) *Biochemistry* **28**, 6373–6378] suggests that their binding characteristics are not identical. In the present study we have made use of a set of EGF/TGF α chimaeric molecules to show that the 13A9 antibody blocks receptor binding of ligands with

TGF α sequences, but not of ligands with EGF sequences, in their C-terminal linear regions. Using HaCaT human keratinocyte cells in culture, it was determined that ligands that are able to bind the EGF receptor in the presence of 13A9 are also able to induce calcium release from intracellular stores in these cells, indicating that these ligands have the ability to activate the EGF receptor in the presence of the antibody. From these data it is concluded that the flexible C-terminal linear domains of EGF and TGF α bind to separate sequences on the EGF receptor, such that the binding domain of TGF α , but not that of EGF, overlaps with the binding epitope of the 13A9 antibody.

INTRODUCTION

Epidermal growth factor (EGF) and transforming growth factor- α (TGF α) belong to a family of structurally related growth factors that exert their action by binding to the EGF receptor (EGF-R) [1]. Human (h)EGF and hTGF α bind with similar affinities to the human, mouse and rat EGF-Rs [2], whereas the chicken EGF-R has been shown to bind hTGF α with a 100-fold higher affinity compared with hEGF binding [3]. Structural studies using high-field NMR techniques have indicated that EGF and TGF α have almost identical polypeptide chain folds, providing a molecular basis for the similar binding characteristics of the two growth factors [4]. Consequently, EGF and TGF α induce similar mitogenic effects in most cells, although a number of cellular systems have been described in which TGF α appears to be more potent than EGF [5,6].

There is still controversy in the literature as to whether EGF and TGF α bind to similar domains on the EGF-R. The observation that EGF and TGF α bind their cognate receptor in a similar competitive fashion, in combination with the structural similarity between them, has led to the hypothesis that both growth factors bind to a similar site on the EGF-R. However, the fact that EGF-like growth factors display only limited sequence identity could indicate that these ligands do not necessarily bind their receptor in an identical manner. Winkler et al. [7] have characterized an antibody against the hEGF-R, designated 13A9, that inhibits binding of TGF α , but not of EGF, indeed suggesting that the binding characteristics of these two growth factors are not identical. Since EGF and TGF α mutually compete for binding, it could either be that these ligands bind to distinct, but partly overlapping, epitopes on the receptor, or that the antibody stabilizes a conformation in the EGF-R that favours binding of

EGF but not of TGF α . The first option is supported by the observation that insertions have been introduced into the extracellular domain of the EGF-R that reduce the binding of TGF α , but not that of EGF [8].

Domain exchange studies between the hEGF-R and the chicken EGF-R pinpointed the main ligand binding domain in the receptor to a region between the two cysteine-rich domains of the extracellular part of the receptor [3]. Antibodies against the hEGF-R that compete with EGF for receptor binding have also been shown to interact with this so-called subdomain III of the receptor in particular [9]. Using such antibodies in cross-linking studies, the region between amino acids 352 and 366 within this subdomain has been implicated directly in ligand binding [10]. Cross-linking studies with iodinated EGF itself have indicated, however, that the C-terminal region of EGF is in direct contact with Lys-465 in subdomain III [11], while the N-terminus is proximal of Tyr-101 in subdomain I of the hEGF-R [12].

We have constructed a set of hEGF/hTGF α chimaeras by exchanging domains bordered by the six conserved cysteine residues of these growth factor molecules [13]. Using these chimaeras, we have shown previously that the C-terminal linear regions of EGF and TGF α , and in particular Arg-45 [13,14], are responsible for the difference in affinities of these two wild-type ligands for the hEGF-R and the chicken EGF-R, thereby demonstrating that this C-terminal region is part of the receptor binding domain. In the present study we have used the same set of chimaeras to investigate which domains in EGF and TGF α are involved in the differential binding of these two growth factors to the hEGF-R in the presence of the 13A9 antibody. Our results show that chimaeras with TGF α C-terminal sequences cannot bind the hEGF-R in the presence of this antibody, whereas chimaeras with EGF C-terminal sequences can do so.

Abbreviations used: EGF, epidermal growth factor; TGF α , transforming growth factor- α ; EGF-R, EGF receptor; DMEM, Dulbecco's modified Eagle's medium; the prefixes h and m denote human and mouse respectively.

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This indicates that EGF and TGF α bind differently to the hEGF-R, mainly because their C-terminal linear domains interact with different regions on the receptor.

MATERIALS AND METHODS

Materials

[³H]Thymidine was obtained from Amersham International, mouse EGF (mEGF) from Bioproducts for Science Inc. (Indianapolis, IN, U.S.A.), and the anti-hEGF-R monoclonal antibody 13A9 from Genentech Inc. (South San Francisco, CA, U.S.A.).

Expression and purification

Chimaeric ligands were constructed and cloned into the pEZZmp18 expression vector (Pharmacia, Uppsala, Sweden) as described previously [13,14]. Recombinant and wild-type ligands were expressed as Protein A-factor X-growth factor fusion proteins [13] and secreted into the periplasmic space of the degP protease-deficient *Escherichia coli* strain KS474 (a gift from Dr. K. L. Strauch and Dr. J. Beckwith, Dept. of Microbiology and Molecular Genetics, Harvard University Medical School, Cambridge, MA, U.S.A.) [15]. Bacteria were grown overnight in 2YTE medium [per litre: 16 g of bactotryptone, 10 g of yeast extract, 8 g of NaCl and 0.4% (w/v) glucose] at 30 °C with agitation (200 rev./min).

Obtained fusion proteins were initially separated from the remaining periplasmic proteins using an IgG-Sepharose column (Amersham Pharmacia) and monitored using an ELISA based on binding competition with biotin-labelled Protein A [16]. Growth factors were separated from the Protein A sequence using enzymic factor X cleavage followed by an additional run over the IgG column. Multimeric and inactive forms of growth factor were removed by reverse-phase HPLC on a Deltapak C18 column (Waters Corp., Milford, MA, U.S.A.) at a flow rate of 1 ml/min, using a linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid, as described previously [14]. Fractions of 1 ml were collected and tested for growth factor activity in a binding competition study in HER-14 cells using radiolabelled mEGF. Finally, the quantity of growth factor was calculated by determining the peak area at 229 nm in the HPLC profile, using fully active natural mEGF as a standard under the same experimental conditions [14].

Cell culture

NIH-3T3 fibroblasts transfected with wild-type hEGF-R (HER-14 cells; see [17]) were provided by Dr. J. Schlessinger (New York University, New York, NY, U.S.A.). HER-14 cells were cultured using gelatin-coated flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum. A non-transfected human keratinocyte cell line expressing the wild-type hEGF-R (HaCaT; see [18]) was donated by Dr. N. E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum.

¹²⁵I-mEGF binding competition assays

mEGF was iodinated using Enzymobeads (Bio-Rad) to a specific radioactivity of approx. 500 Ci/mmol [19]. HER-14 and HaCaT cells were grown to confluence in gelatin-coated 96-well dishes (0.33 cm²). The medium was then removed and serial dilutions of recombinant growth factors were added in 25 μ l of DMEM containing 15 mM Hepes, pH 7.7, and 10% (v/v) newborn calf

serum (DMEM/Hepes), together with 1 ng/ml ¹²⁵I-mEGF, in the presence or absence of 1.5 μ g/ml 13A9 antibody. After an incubation of 2 h at room temperature, the cells were washed twice with ice-cold PBS supplemented with 0.1% BSA (PBS/BSA) and once with ice-cold PBS. Subsequently cells were lysed in 100 μ l of 1% (v/v) Triton X-100/well for 1 h at room temperature prior to γ -radiation counting.

Intracellular Ca²⁺ measurements

HaCaT cells were grown on gelatin-coated glass coverslips and subsequently loaded with 2 μ M fura2-AM (Molecular Probes, Eugene, OR, U.S.A.) in calcium- and serum-free bicarbonate-buffered medium containing 0.01% (w/v) Pluronic F127 (Molecular Probes) for 30 min. After loading, the cells were washed with serum-free medium for 15 min, and the 13A9 antibody was added to the cells in 1 μ l of Bes-buffered (50 mM, pH 7.0) DMEM to a final concentration of 1.5 μ g/ml. After 1 min of incubation, recombinant growth factor was added in 4 μ l to a final concentration of 40 ng/ml. Excitation wavelengths were 340 and 380 nm, while fluorescence emission was monitored at 492 nm. Dynamic video imaging was carried out using the MagiCal hardware and Tardis software of Joyce Loebel (Tyne and Wear, U.K.), as described previously [20]. All procedures were carried out at room temperature.

RESULTS

Antibody 13A9 blocks binding of TGF α , but not of EGF, to the hEGF-R

In order to verify the specificity of the 13A9 antibody in the cells used in this study, binding of ¹²⁵I-mEGF to HER-14 cells was determined in the presence of increasing concentrations of the antibody. HER-14 cells are NIH-3T3 cells transfected with the hEGF-R; this cell line contains approx. 4.0×10^5 hEGF-Rs per cell [17]. Figure 1 shows that, up to concentrations of approx. 5 μ g/ml, the antibody did not significantly affect binding of radiolabelled mEGF, indicating that, in contrast with other anti-hEGF-R antibodies, 13A9 does not inhibit binding of EGF to a specific subset of its receptors [21]. Figure 1 also shows that unlabelled EGF and TGF α are both able to compete with radiolabelled EGF in the absence of the antibody; however, upon addition of the antibody, competition by EGF is retained while that by TGF α is lost, in agreement with the original data of Winkler et al. [7]. Moreover, the results indicate that a concentration of the antibody of 1 μ g/ml is sufficient to prevent TGF α binding to the hEGF-R under these conditions. Similar results were obtained (not shown) when using the non-transfected HaCaT human keratinocyte cell line, which expresses approx. 3.0×10^4 wild-type hEGF-Rs per cell [18].

Selective effect of the 13A9 antibody on binding of EGF/TGF α chimaeras to the hEGF-R

Using the set of EGF/TGF α chimaeras previously characterized by us [13], we set out to study which domains in TGF α are involved in its inability to bind the hEGF-R in the presence of the 13A9 antibody. Figure 2 shows the effects of the addition of 1.5 μ g/ml 13A9 on the ability of EGF, TGF α and EGF/TGF α chimaeras to compete with binding of radiolabelled EGF to HER-14 cells (Figures 2A–2C) and HaCaT cells (Figures 2D–2F). The chimaeras have been designated according to their previously described nomenclature; for example, E3T stands for a ligand with hEGF sequences N-terminal and TGF α sequences C-terminal to the third cysteine [13].

Figure 1 Effect of the hEGF-R blocking antibody 13A9 on binding of ^{125}I -mEGF

HER-14 cells were grown to confluency and incubated for 2 h at room temperature with 1 ng/ml ^{125}I -mEGF together with the indicated concentrations of 13A9 in the absence (upper panel) or presence (lower panel) of 100 ng/ml EGF (■) or TGF α (□). Cell surface binding of radiolabelled ligand is given as the mean $\pm$ S.E.M. from a triplicate experiment and is expressed relative to the amount of binding in the absence of both antibody and unlabelled ligand.

In the absence of the 13A9 antibody, all ligands tested had similar binding affinities for the hEGF-R (Figures 2A and 2D), in agreement with our previous data [14]. Figures 2(B) and 2(E) show that not only TGF α , but also those chimaeras with TGF α sequences in their linear C-terminal region (E3T, E4T and E6T), were unable to compete with EGF in the presence of the antibody. Figures 2(C) and 2(F) show that, in contrast, EGF and chimaeras with EGF sequences in their C-terminal linear region (T3E, T4E and T6E) were able to compete with binding under these conditions. The data presented show that T3E, T4E and hEGF have similar high binding affinities in both the absence and the presence of 13A9, whereas T6E has an intermediate binding affinity in the presence of the antibody.

These results indicate that the linear C-terminal regions of EGF and TGF α in particular determine whether EGF-like ligands are able to bind their cognate receptor in the presence of the 13A9 antibody. However, the somewhat lower binding affinity of T6E compared with T4E suggests that amino acids in the so-called C-loop [22] of these growth factors may also play a role.

13A9 antibody blocks calcium responses in HaCaT cells

Activation of growth factor receptors can result in a rapid transient increase in the intracellular calcium concentration due to calcium release from internal stores. Figures 3(A)–(D) show that, in the absence of antibody 13A9, TGF α , EGF, E6T and T6E all induced an increase in intracellular calcium in HaCaT cells within 5 min of beginning the incubation with the indicated

Figure 2 Effect of 13A9 antibody on the binding of EGF/TGF α chimaeras

Binding of ^{125}I -mEGF to HER-14 (A–C) and HaCaT (D–F) cells was performed as described in the legend to Figure 1, in the presence of the indicated concentrations of hEGF (▼), hTGF α (▽), E3T (△), T3E (▲), E4T (○), T4E (●), E6T (□) or T6E (■), and in the absence (A and D) or presence (B, C, E and F) of 1.5 $\mu\text{g/ml}$ 13A9. Results are given as the means $\pm$ S.E.M. of three independent experiments carried out in triplicate. See the Results section for a description of chimaera nomenclature.

Figure 3 Mobilization of intracellular calcium by EGF/TGF α chimaeras

HaCaT cells were grown on gelatin-coated coverslips, loaded with fura2 and subsequently stimulated to release intracellular Ca^{2+} by adding 40 ng/ml hTGF α , hEGF, E6T or T6E (A–D; arrow). The effect of the 13A9 antibody (Ab) on calcium release was determined by preincubating the fura-loaded keratinocytes with 1.5 $\mu\text{g}/\text{ml}$ 13A9 antibody (E–H; first arrow) for 1 min prior to ligand addition (second arrow).

ligand. Unlike in previous reports [23], no significant calcium response was observed upon treatment of HER-14 cells with these various ligands (results not shown). When HaCaT cells were pretreated with 13A9, EGF was still able to produce a calcium response, in agreement with previous data in A431 cells [24], but under the same conditions TGF α was unable to do so (Figures 3E and 3F). This shows that inhibition of TGF α binding by the antibody also results in a blockade of TGF α -induced second messengers. With the chimaeras E6T and T6E, pretreatment with 13A9 largely blocked the calcium response induced by E6T, but not that induced by T6E (Figures 3G and 3H). These results indicate that EGF-like growth factors that are able to bind the hEGF-R in the presence of antibody 13A9 are also able to activate this receptor and to generate intracellular second messengers.

DISCUSSION

In the present study we have shown, using hEGF/hTGF α chimaeras, that the 13A9 antibody blocks receptor binding of EGF-like growth factors that have TGF α sequences in their C-terminal linear regions. This indicates that the linear C-terminal regions in particular of EGF and TGF α bind to different epitopes on the EGF-R. This would imply that the EGF/TGF α chimaeras would have to make a 'choice' to bind either to the EGF epitope

or to the TGF α epitope, or to form a bridge between the two epitopes. Obviously a chimaera such as T6E does not bind to the TGF α epitope, since its binding is only poorly affected by the 13A9 antibody. On the other hand, it seems unlikely that the linear C-terminal domain is sufficient to direct T6E to the EGF epitope. The observation that all ligands that are able to bind the hEGF-R in the presence of the antibody are also able to induce a calcium response (Figure 3) implies that these factors not only are able to bind the receptor, but are also able to activate it. This makes it unlikely that T6E binds only with its C-terminal domain to the EGF epitope without bridging to the TGF α epitope. Based on these data, we favour a model in which the looped areas of EGF and TGF α bind to a similar site on the hEGF-R outside the binding domain of the 13A9 antibody. However, the flexible linear C-terminal regions of EGF and TGF α bind to separate sequences on the receptor, in such a way that the binding domain of TGF α , but not that of EGF, interferes with the binding epitope of 13A9.

Previous data obtained by Carraway and Cerione [24] showed that 13A9 binding prevents EGF-induced receptor dimerization in A431 cells. In spite of this, 13A9 does not block EGF-induced receptor tyrosine kinase activity or increases in the intracellular calcium concentration in these cells. The observation in the present study that 13A9 does not affect the binding affinity of EGF, T3E or T4E would therefore imply that the affinity of EGF-like growth factors for their receptor is not affected by the ability of the receptor to dimerize. On the other hand, increasing evidence shows that binding of EGF-like growth factors to the ErbB-1 receptor may result not only in the formation of ErbB-1 homodimers, but also in the formation of receptor heterodimers, of which particularly those with ErbB-2 have strong signalling activity [25,26]. It has been established that, in A431 cells, EGF is able to induce the formation of both ErbB-1 homodimers and ErbB-1/ErbB-2 heterodimers [27]. The possibility therefore cannot be excluded that the 13A9 antibody is able to prevent the formation of EGF-induced ErbB-1 homodimers, but not of ErbB-1/ErbB-2 heterodimers, resulting in the EGF-induced receptor tyrosine kinase activity and enhancement of intracellular calcium concentration observed by Carraway and Cerione [24].

Many studies have been performed on the structure–function relationships of hEGF and TGF α . Based on site-directed mutagenesis [22] and structural NMR studies [28], evidence has been presented that, besides conserved cysteine and glycine residues that are required for a proper three-dimensional structure, additional amino acids are important for direct interaction with the receptor. These include the residues located around the second cysteine (Tyr-13, Leu-15 and His-16), around the sixth cysteine (Tyr-37, Arg-41 and Gln-43) and in the linear C-terminal tail (Leu-47) of hEGF. Our previous results [14] have indicated the importance of Arg-45 in EGF-R binding, while our present results emphasize the specific role of the linear C-terminal tail in receptor recognition. This agrees with recent NMR data on complexes of TGF α with the extracellular domain of the EGF-R, which show a direct involvement of the linear C-terminal tail in receptor binding [29]. Interestingly hEGF is a more acidic molecule than TGF α , but the linear C-terminal region of hEGF is basic, in contrast with that of TGF α [30]. Further exchange studies between hEGF and TGF α will help to identify which amino acids in this linear C-terminal region are of direct importance in receptor recognition.

We thank Dr. T. J. Benraad and Mr. J. J. T. M. Heuvel (Department of Experimental and Chemical Endocrinology, University Hospital St. Radboud, The Netherlands) for the generous supply of ^{125}I -mEGF, and Genentech Inc. for the 13A9 antibody. The

present studies were carried out with financial support from the Dutch Cancer Society (KWF 93-493).

REFERENCES

- 1 Prigent, S. A. and Lemoine, N. R. (1992) *Prog. Growth Factor Res.* **4**, 1–24
- 2 Massagué, J. (1983) *J. Biol. Chem.* **258**, 13614–13620
- 3 Lax, I., Bellot, F., Howk, R., Ullrich, A., Givol, D. and Schlessinger, J. (1989) *EMBO J.* **8**, 421–427
- 4 Campbell, I. D., Cooke, R. M., Baron, M., Harvey, T. S. and Tappin, M. J. (1989) *Prog. Growth Factor Res.* **1**, 13–22
- 5 Schreiber, A. B., Winkler, M. E. and Derynck, R. (1986) *Science* **232**, 1250–1253
- 6 Barrandon, Y. and Green, H. (1987) *Cell* **50**, 1131–1137
- 7 Winkler, M. E., O'Connor, L., Winget, M. and Fendly, B. (1989) *Biochemistry* **28**, 6373–6378
- 8 Harte, M. T. and Gentry, L. E. (1995) *Arch. Biochem. Biophys.* **322**, 378–389
- 9 Lax, I., Fischer, R., Ng, C., Segre, J., Ullrich, A., Givol, D. and Schlessinger, J. (1991) *Cell Regul.* **2**, 337–345
- 10 Wu, D. G., Wang, L. H., Sato, G. H., West, K. A., Harris, W. R., Crabb, J. W. and Sato, J. D. (1989) *J. Biol. Chem.* **264**, 17469–17475
- 11 Summerfield, A. E., Hudnall, A. K., Lukas, T. J., Guyer, C. A. and Staros, J. V. (1996) *J. Biol. Chem.* **271**, 19656–19659
- 12 Woltjer, R. L., Lucas, T. J. and Staros, J. V. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7801–7805
- 13 Kramer, R. H., Lenferink, A. E. G., Lammerts van Bueren-Koornneef, I., van der Meer, A., van de Poll, M. L. M. and van Zoelen, E. J. J. (1994) *J. Biol. Chem.* **269**, 8707–8711
- 14 van de Poll, M. L. M., Lenferink, A. E. G., van Vugt, M. J. H., Jacobs, J. J., Janssen, J. W., Joldersma, M. and van Zoelen, E. J. J. (1995) *J. Biol. Chem.* **270**, 22337–22343
- 15 Strauch, K. L., Johnson, K. and Beckwith, J. (1989) *J. Bacteriol.* **171**, 2689–2696
- 16 Van Zoelen, E. J., Kramer, R. H., van Reen, M. M., Veerkamp, J. H. and Ross, H. A. (1993) *Biochemistry* **32**, 6275–6280
- 17 Honegger, A., Dull, T. J., Bellot, F., Van Obberghen, E., Szapary, D., Schmidt, A., Ullrich, A. and Schlessinger, J. (1988) *EMBO J.* **7**, 3045–3052
- 18 Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N. E. (1988) *J. Cell Biol.* **106**, 761–771
- 19 Kienhuis, C. B., Heuvel, J. J., Ross, H. A., Swinkels, L. M., Foekens, J. A. and Benraad, T. J. (1991) *Clin. Chem.* **37**, 1749–1755
- 20 Willems, P. H., Van Emst-De Vries, S. E., Van Os, C. H. and De Pont, J. J. (1993) *Cell Calcium* **14**, 145–159
- 21 Defize, L. H., Boonstra, J., Meisenhelder, J., Kruijer, W., Tertoolen, L. G., Tilly, B. C., Hunter, T., van Bergen en Henegouwen, P. M., Moolenaar, W. H. and de Laat, S. W. (1989) *J. Cell Biol.* **109**, 2495–2507
- 22 Groenen, L. C., Nice, E. C. and Burgess, A. W. (1994) *Growth Factors* **11**, 235–257
- 23 Schalkwijk, C. G., van der Heijden, M. A., Bunt, G., Maas, R., Tertoolen, L. G., van Bergen en Henegouwen, P. M., Verkleij, A. J., van den Bosch, H. and Boonstra, J. (1996) *Biochem. J.* **313**, 91–96
- 24 Carraway, K. L. and Cerione, R. A. (1993) *J. Biol. Chem.* **268**, 23860–23867
- 25 Karunakaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E. and Yarden, Y. (1996) *EMBO J.* **15**, 254–264
- 26 Graus-Porta, D., Beerli, R., Daly, J. M. and Hynes, N. E. (1997) *EMBO J.* **16**, 1647–1655
- 27 Huang, G. C., Ouyang, X. and Epstein, R. J. (1988) *Biochem. J.* **331**, 113–119
- 28 Hommel, U., Harvey, T. S., Driscoll, P. C. and Campbell, I. D. (1992) *J. Mol. Biol.* **227**, 271–282
- 29 McInnes, C., Hoyt, D. W., Harkins, R. N., Pagila, R. N., Debanne, M. T., O'Connor-McCourt, M. and Sykes, B. D. (1996) *J. Biol. Chem.* **271**, 32204–32211
- 30 Lenferink, A. E., Kramer, R. H., van Vugt, M. J., Konigswieser, M., Di Fiore, P. P., van Zoelen, E. J. and van de Poll, M. L. (1997) *Biochem. J.* **327**, 859–865