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Epidermal growth factor binding induces a conformational change in the external domain of its receptor

C.Greenfield, I.Hiles, M.D.Waterfield, M.Federwisch[1], A.Wollmer, T.L.Blundell[2] and N.McDonald[2]

Ludwig Institute for Cancer Research, Middlesex Branch, London W1P 8BT, London, [1]Institute für Biochemie, Rheinisch-Westfalische Technische Hochscule, Aachen, FRG and [2]Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, University of London, Malet Street, London WC1 E7HX, UK

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To study the properties of the extracellular epidermal growth factor (EGF) binding domain of the human EGF receptor, we have infected insect cells with a suitably engineered baculovirus vector containing the cDNA encoding the entire ectodomain of the parent molecule. This resulted in a correctly folded, stable, 110 kd protein which possessed an EGF binding affinity of 200 nM. The protein was routinely purified in milligram amounts from 1 litre insect cell cultures using a series of three standard chromatographic steps. The properties of the ectodomain were studied before and after the addition of different EGF ligands, using both circular dichroism and fluorescence spectroscopic techniques. A secondary structural analysis of the far UV CD spectrum of the ectodomain indicated significant proportions of α-helix and β-sheet in agreement with a published model of the EGF receptor. The ligand additions to the receptor showed differences in both the near- and far-UV CD spectra, and were similar for each ligand used, suggesting similar conformational differences between uncomplexed and complexed receptor. Steady-state fluorescence measurements indicated that the tryptophan residues present in the ectodomain are buried and that the solvent-accessible tryptophans in the ligands become buried on binding the receptor. The rotational correlation times measured by fluorescence anisotropy decay for the receptor-ligand complexes were decreased from 6 to 2.5 ns in each case. This may indicate a perturbation of the tryptophan environment of the receptor on ligand binding. Ultracentrifugation studies showed that no aggregation occurred on ligand addition, so this could not explain the observed differences from CD or fluorescence. We therefore conclude that the measured differences are a result of a specific ligand-induced conformational change in the receptor and/or the EGF ligand.

Key words: baculovirus/EGF receptor/purification/solution structure/spectroscopy

Introduction

Epidermal growth factor (EGF) and transforming growth factor alpha (α-TGF) are polypeptide mitogens for epithelial and mesenchymal target cells. The biological effects of these growth factors are mediated by interaction with a specific cell surface glycoprotein receptor (reviewed in Carpenter, 1987; Yarden and Ullrich, 1988). The receptor consists of an external ligand-binding domain separated from a cytoplasmic domain by a 20 amino-acid transmembrane stretch (Ullrich *et al.*, 1984). Ligand binding to the receptor external domain stimulates an intrinsic tyrosine protein kinase activity which is associated with the cytoplasmic domain of the receptor (reviewed in Yarden and Ullrich, 1989).

Two allosteric mechanisms have been suggested to explain the signal transduction process. The proposed intramolecular mechanism involves a ligand-induced conformational change in the receptor extracellular domain which can be transmitted through the transmembrane region and results in the activation of the tyrosine protein kinase activity of the receptor (Staros *et al.*, 1985; Koland and Cerione, 1988). The alternative intermolecular mechanism involves ligand-induced stabilization of an oligomeric, most likely dimeric state of the complex, which possesses an elevated kinase activity (Yarden and Schlessinger 1987a,b; Schlessinger, 1988). Both models require that a conformational change is initiated by binding of ligand to the receptor. Recent work (Lax *et al.*, 1988, 1989) has indicated that the L_2 domain (see Bajaj *et al.*, 1987), which contains residues 333-460, forms a major part of the EGF binding site. To detect conformational changes in the receptor and to understand the molecular interactions between ligand and receptor, detailed structural studies using spectroscopic and X-ray crystallographic methods are ideally required. Such experiments require milligram quantities of purified receptor or receptor domains, which retain high-affinity binding sites for the ligand. It is important that the system utilized should be amenable to the use of site-directed mutagenesis studies for the evaluation of the predictions made by the two models.

Efficient purification schemes have been developed for the isolation of homogeneous EGF receptor from natural sources such as human placenta or the A431 cell line (for a review see Panayotou *et al.*, 1989); however, these systems cannot be used for mutagenesis studies. We have previously reported the successful expression of a full-length human EGF receptor in insect cells (Greenfield *et al.*, 1988). This was produced using a baculovirus expression system utilizing the polyhedrin promoter (Summers and Smith, 1987; Smith *et al.*, 1983) and resulted in the expression of a correctly orientated receptor with respect to the plasma membrane which possessed a ligand stimulatable tyrosine kinase activity. However, it has not yet been possible to purify adequate quantities of this recombinant membrane protein with an active kinase because conventional purification methods result in denaturation. In the present study we have chosen to express only the external domain of the EGF receptor as a secreted product using the insect cell system because it is more amenable to physico-chemical characterization without the detergent requirements for

solubilization needed for integral membrane proteins. Our rationale for this approach was based on three lines of evidence that each domain can function autonomously. Firstly, as a result of a gene rearrangement, A431 carcinoma cells express a 117 kd EGF binding protein which corresponds to the external domain of the EGF receptor (Ullrich *et al.*, 1984). Secondly, the extracellular domain of the insulin receptor has been expressed, and maintains its ligand binding properties (Ellis *et al.*, 1988; Whittaker and Okamoto, 1988). Thirdly, the protein molecule encoded for by a chimeric cDNA corresponding to the human EGF receptor external domain in frame with the transmembrane and cytoplasmic domain encoded by the v-*erbB* gene has been shown to bind EGF (Riedel *et al.*, 1987).

To study the solution conformation of the secreted EGF receptor external domain and the consequence of ligand binding, a spectroscopic analysis of these molecules was performed using circular dichroism (CD) and fluorescence. Both techniques are non-destructive and are sensitive indicators of changes in protein conformation (Bayley, 1973; Curtiss Johnson, 1988; Lakowicz, 1986) even though a specific structural interpretation cannot always be made (Skehel *et al.*, 1982; Turkewitz *et al.*, 1988). These methods can also be applied to provide limited information on receptor structure in solution (Cheng *et al.*, 1972; Philips *et al.*, 1989).

Here the production of milligram amounts of the external domain of the EGF receptor using insect cells is described. The domain is shown to be stable, has a high affinity for EGF, but does not undergo aggregation or dimerization after ligand addition. We then demonstrate that changes in the solution conformation of the receptor can be detected after the addition of different ligands. Two different ligands (human EGF and human α-TGF) were used for the CD measurements and three different ligands (human EGF, mouse EGF and human α-TGF) for the fluorescence measurements. We interpret these observed changes to be the consequence of a specific EGF-induced conformational change in the receptor and/or the bound ligand.

Results

Expression of the external domain

The strategy used for the assembly of the insect vector containing the coding sequence for the extracellular domain of the EGF receptor is somewhat complicated and is outlined below. The specifications required of this vector were that it should efficiently express a secreted product, thereby simplifying subsequent protein purification. The plasmid A8 contains all the 5′ non-coding sequences of the polyhedrin gene and also includes the authentic first five 5′ coding nucleotides (ATGCG), thereby leaving intact the polyhedrin sequences thought to be responsible for efficient gene transcription (Summers and Smith, 1987). The rest of the coding sequence comprises the external domain of the EGF receptor (including the signal sequence). This contains 622 residues, of which only Pro621 which is changed to Leu and the Ser622 residue (the last before the putative transmembrane region) which is replaced by a stop codon, are different from the corresponding sequence in the full length human receptor. The co-transfection of the receptor construct and the wild-type virus into Sf9 cells and the identification of recombinant virus was performed as previously described (Greenfield *et al.*, 1988); the virus was then used for infecting 1 l volumes of culture medium containing the insect cells.

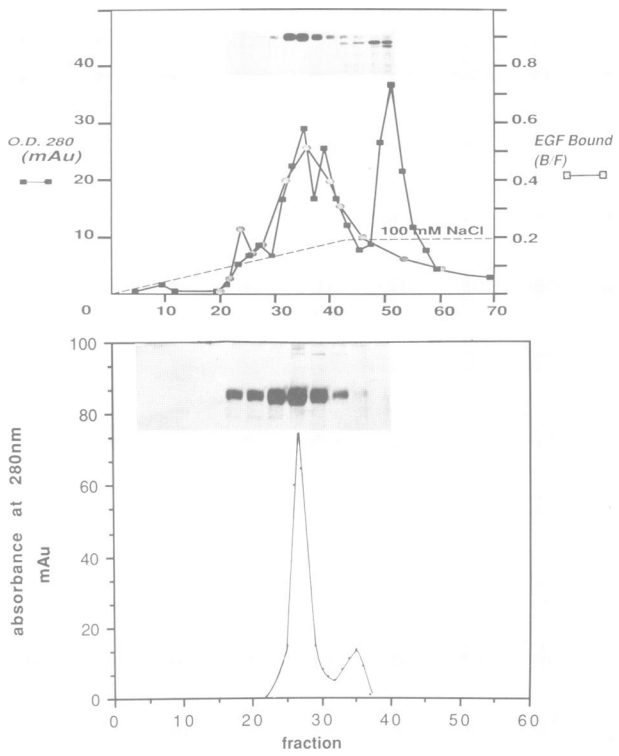

Fig. 1. Purification of extracellular EGF receptor domain. The receptor was purified by lentil lectin-agarose and MonoQ ion exchange (**a**) and was then chromatographed on a Superose 12 column (**b**). (**Insets**) Silver staining of the receptor by SDS−PAGE under reducing conditions of a) fractions 31−51 of the Mono Q column and b) fractions 24−33 of the Superose run.

Purification of the external domain

The external domain of the receptor was secreted into the insect medium, which was collected 4 days post-infection, centrifuged to remove cellular debris and passed through a lentil lectin column to obtain a glycoprotein fraction. This step also achieved a 5- to 10-fold concentration of the receptor domain. The glycoprotein fraction was then further purified by anion-exchange chromatography using a Mono Q FPLC column with a salt gradient from which the receptor external domain eluted at 75−100 mM NaCl. Two absorbance peaks and a single EGF binding peak were detected (Figure 1a). The nature of these two peaks is not known at present, but the fractions from both were pooled for further purification (fractions 31−41). After concentration and dialysis, the material was subjected to gel exclusion chromatography on a Superose 12 FPLC column, where the receptor domain eluted with an apparent mol. wt of 110 000 as judged by SDS−PAGE analysis of fractions (see Figure 1b). This figure is in good agreement with the calculated mass of 92−98 000 daltons. The column chromatography step using Superose was also important for removal of azide, which would have interfered with CD measurements. The overall purification yielded 1 mg of purified receptor from 5×10^9 cells or ~1 l of cells. The amino-terminal sequence of this purified material was then determined and

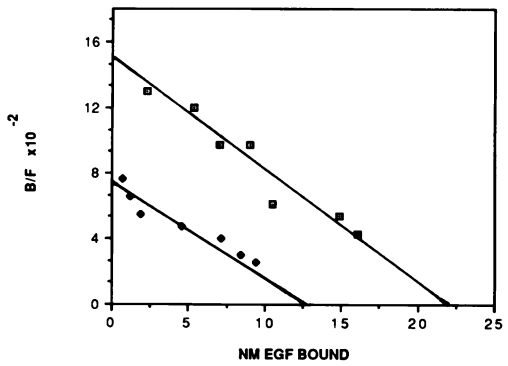

Fig. 2. [^{125}I]EGF binding to the extracellular EGF receptor (■) as described in Materials and methods. EGF competition with [^{125}I]EGF binding to the secreted A431 cell line EGF receptor (◆) is shown for comparison.

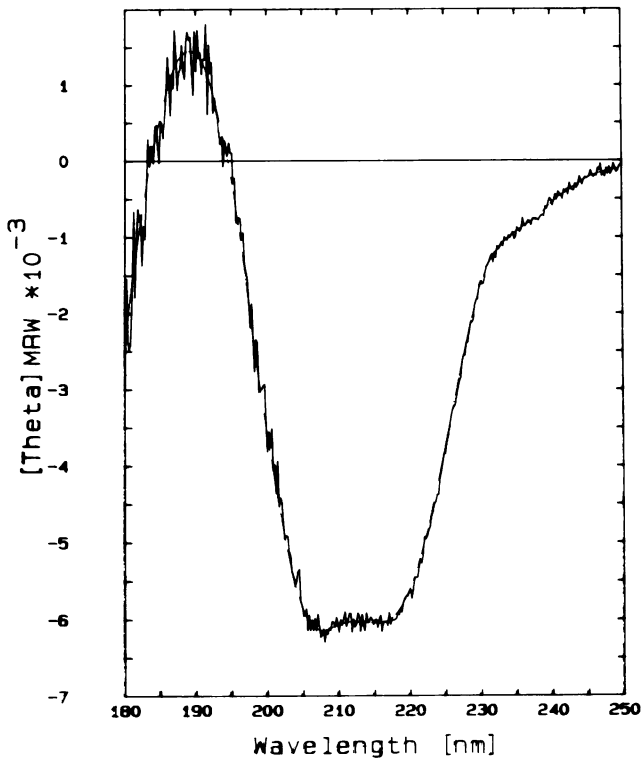

Fig. 3. The far-UV CD of the EGF receptor external domain. An original tracing is shown including noise. Samples were prepared and collected as described in Materials and methods.

found to be identical to that of the mature EGF receptor as previously determined by Downward *et al.* (1984).

Ligand binding assay

Receptor binding of the HPLC-purified growth factors human EGF and α-TGF was assessed by their ability to inhibit the binding of ^{125}I-labelled EGF to A431 cells. Binding affinity was found to be identical to that of a mouse EGF standard purified by the classical Cohen protocol (Savage *et al.*, 1972). In order to determine whether the receptor domain was functionally active, the binding affinity for EGF was measured and compared to solubilized, purified EGF receptor obtained from A431 cells (Downward *et al.*, 1984). The K_d as determined by Scatchard analysis for both the full-length solubilized receptor and the external domain were very similar: 2×10^{-7} and 3.5×10^{-7} M respectively (see Figure 2).

Table I. Secondary structure prediction by sequence and circular dichroism analysis

Method	Helix	β-sheet	Other
Burgess	4%	26%	32%
Dufton	16%	38%	19%
Chou and Fasman	41%	27%	25%
Garner	23%	26%	40%
Lim	31%	19%	–
Joint	21%	24%	33%
CD analysis	15 ± 0.9%	53 ± 1%	32 ± 1.7%

The secondary sequence predictions were performed using the Leeds prediction package (Eliopoulos, 1989), with a joint prediction resulting from the averaging of all five methods. The CD analysis was performed using CONTIN as described in Materials and method and Provencher and Glockner (1981).

Circular dichroism

An original tracing of the CD spectrum of the EGF receptor ectodomain is shown in Figure 3. Measurements made on the same material at a later date, or on different preparations, were also very similar, suggesting that the protein was stable and that there was little variation between individual preparations. The far-UV spectrum (Figure 3) is characterized by three extrema, with minima at 217 and 208 nm and a maximum at 190 nm—with the baseline crossed at 195 and 183 nm. The minimum at 208 nm has a slightly larger intensity than at 217 nm, but each has a magnitude approximately four times greater than the 190 nm maximum. An analysis of this CD spectrum by the CONTIN program resulted in a secondary structrual composition of 15% α-helix, 53% β-sheet and 32% non-periodic structure. This can be compared with the joint structure prediction deduced from analysis of amino acid sequence which is shown in Table I. Whilst the helical and non-periodic contributions predicted are in reasonable agreement with those derived from the CD analysis, there is a large disparity for the contribution of β-sheet (24% compared with 53%). The near-UV spectrum is negative throughout, with minima at 275 and 298 nm and a maximum at 290 nm (see Figure 5).

The far-UV CD spectra of recombinant human EGF and α-TGF are both dominated by a negative band at 200 nm but with different rotational strengths, reflecting the preponderance of non-periodically ordered structure. The absence of signals characteristic of α-helix is expected, based on results from recent two-dimensional NMR studies (Cooke *et al.*, 1987; Montelione *et al.*, 1987).

The far-UV CD spectra of the free receptor, of the free ligand and of the ligand–receptor complex after subtraction of the CD spectrum of the free ligand at the concentration present in the mixture are shown in Figure 4. Small but reproducible differences are seen for human EGF (Figure 4a) and α-TGF (Figure 4b) across the wavelength range covered suggesting that these differences observed for EGF are a direct consequence of the binding of EGF to its receptor. The CD experiment with native mouse EGF could not yet be carried out because the amount of material was limited. For the same reason, inclusion of the near-UV data had to be restricted to human EGF. After equimolar additions of human EGF to the receptor, the absolute ellipticity below 280 nm is increased (see Figure 5).

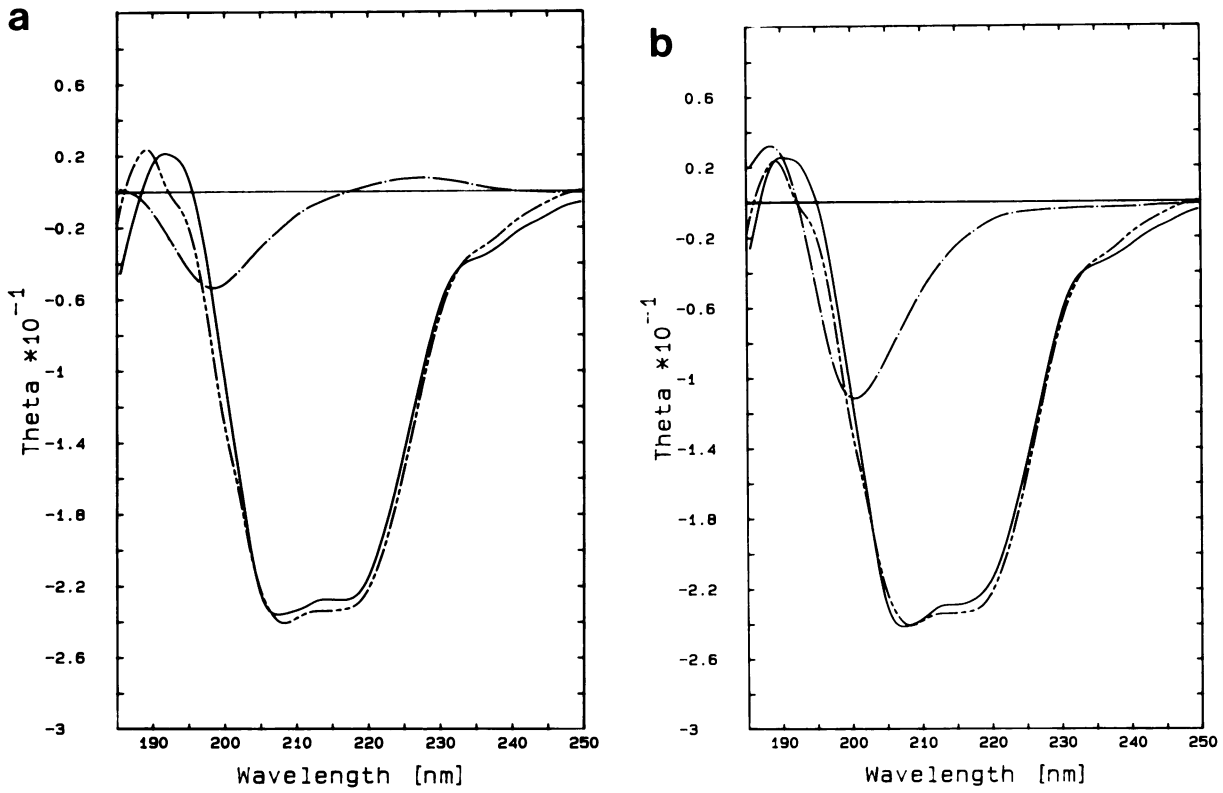

Fig. 4. The smoothed far-UV CD spectrum of the EGF receptor external domain before and after the addition of recombinant human EGF (**a**) and recombinant α-TGF (**b**). The spectra shown are those of the uncomplexed receptor (– · · – · · –), the complexed receptor after subtraction of the ligand CD spectrum (———), and the CD spectrum of the ligand (- · - · - ·) at the concentration present in the mixture. Equimolar ratios of the two ligands were added as described in Materials and methods. The *y*-axis corresponds to ellipticity per cm pathlength, rather than the mean residue weight ellipticity used in Figure 3.

Steady-state and time-resolved fluorescence

The steady-state fluorescence results for both the receptor and receptor complexes are presented in Table II. Both human EGF and the external domain contain tryptophan and tyrosine residues, whereas α-TGF contains only a single tyrosine and no tryptophan. The excitation wavelength used was 295 nm where the contribution of tyrosine to the tryptophan fluorescence is negligible. Using the classification proposed by Burstein *et al.* (1973), the receptor tryptophans would be assigned to class I with a λ_{max} of 333 nm, suggesting that they are buried within the receptor. In contrast, the human EGF contributes two class II tryptophans with a λ_{max} of 344 nm and they must therefore be located on the protein surface. This localization is also suggested by the two-dimensional NMR structure (Cooke *et al.*, 1987). The fluorescence maximum of the receptor remains unchanged after the addition of each growth factor provided that a molar ratio of 1:1 is not exceeded; this suggests that the human EGF tryptophans move into a non-polar environment on binding the receptor where they are shielded from solvent and thus have a shorter λ_{max}. Addition of excess ligand, which is not bound, causes a red shift of the fluorescence maximum to 336 nm, which is towards the λ_{max} of human EGF.

Extension of the steady-state experiments to time-resolved fluorescence can potentially yield more information about the tryptophan environment. Two representative examples of the time-resolved fluorescence measurements are given and show the decays of the total fluorescence intensity (see Figure 6a) and the differences between the polarized

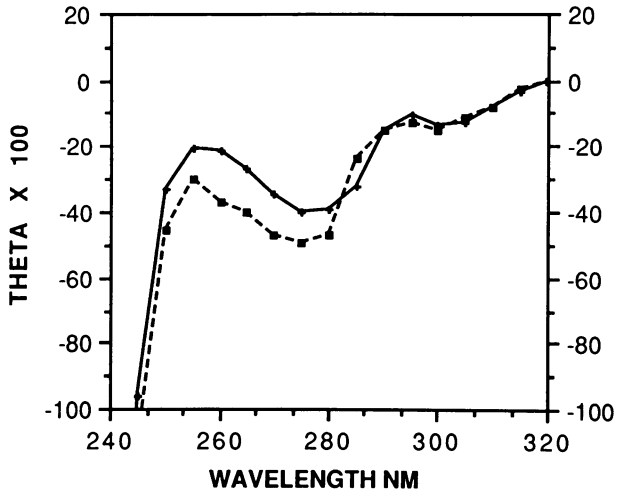

Fig. 5. The smoothed near-UV CD spectrum of the EGF receptor external domain before (———) and after (--------) the addition of equimolar amounts of human EGF.

fluorescence intensities (see Figure 6b) of the receptor. All lifetime data are listed in Table III, which shows three lifetimes (t_i) and their respective amplitudes (b_i). In all cases a sum of these three exponentials was necessary to achieve a good fit with the total fluorescence intensity decay. However, the large number of tryptophans (six in the receptor ectodomain) and the fact that even single tryptophans often exhibit multi-exponential fluorescence

Table II. Details of the steady-state fluorescence measurements including receptor, ligand and receptor–ligand complex

Sample	Emission λ_{max} (nm)	Bandwidth $\Delta\lambda$ (nm)	Relative intensity, I	I/A	$A^{1cm}_{295\ nm}$
Human EGF	344	65	2.4	92.3	0.026
Mouse EGF	346	60	1.7	73.9	0.023
α-TGF[a]	302	45	1.17	13.9	0.084
Receptor	333	55	4.2	95.4	0.044
Receptor:hEGF (1:1)	333	53	4.4	147.0	0.030
Receptor:hEGF (1:2)	336	60	4.75	–	–
Receptor:mEGF (1:1)	333	55	2.47	91.5	0.027
Receptor:α-TGF (1:1)	332	53	3.05	113.0	0.027

[a]The absorbance and fluorescence excitation wavelength was 277 nm.

decays (Eftink and Wasylewski, 1989) meant that a resolution of individual tryptophan fluorescence could not be attempted. A comparison of relative amplitudes and lifetimes indicates the presence of three distinct groups: receptor, ligand and receptor–ligand complex. The anisotropy data obey the same classification. After ligand binding the average rotational correlation time (t_r) of the complex is significantly and consistently lowered from 6.2 to 2.5 ns (see data in Table III). One would usually expect two rotational correlation times to be observed, one for the overall rotational motion of the protein and one for the segmental motion of tryptophan residues within the protein matrix. However, an attempt to fit two rotational correlation times to the observed decay curves led to unstable limiting anisotropy values r_0 and t_r of < 1.0 ns (the resolution limit of the instrument used here), and no reduction of the chi-squared fit (data not shown). The non-zero r_∞ reported in Table III suggests restricted motion of the tryptophans within the receptor matrix. The overall motion of the receptor probably has a $t_r > 20$ ns which would therefore be difficult to measure (Lakowicz and Weber, 1980). This would explain why only a single t_r is seen. Hence it is likely that the observed t_r is an average correlation time for the segmental motion of the buried tryptophan residues within the receptor. It lies within the t_r range of 0.83–12 ns observed for the segmental mobility of tryptophans in proteins (Lakowicz and Weber, 1980). Denaturation is not a possible cause of the reduced t_r, since the limiting anisotropy r_0 would then also be decreased (Lakowicz *et al.*, 1983), however r_0 is actually increased after binding of the ligand. Since these r_0 values are slightly smaller than those measured by Lakowicz *et al.* (1983) for proteins excited at 295 nm a rotational correlation time below 0.1 ns may exist but cannot be resolved (Ichiye and Karplus, 1983).

Ultracentrifugation

The changes observed in the CD and fluorescence measurements on receptor–ligand complex formation suggests that a ligand-induced conformational change occurs in the receptor. However, ligand binding may induce oligomerization of the receptor (Yarden and Schlessinger, 1987a,b) and these oligomers could have a different CD and fluorescence spectrum. In order to determine whether EGF induced oligomerization of the external domain of the EGF receptor,

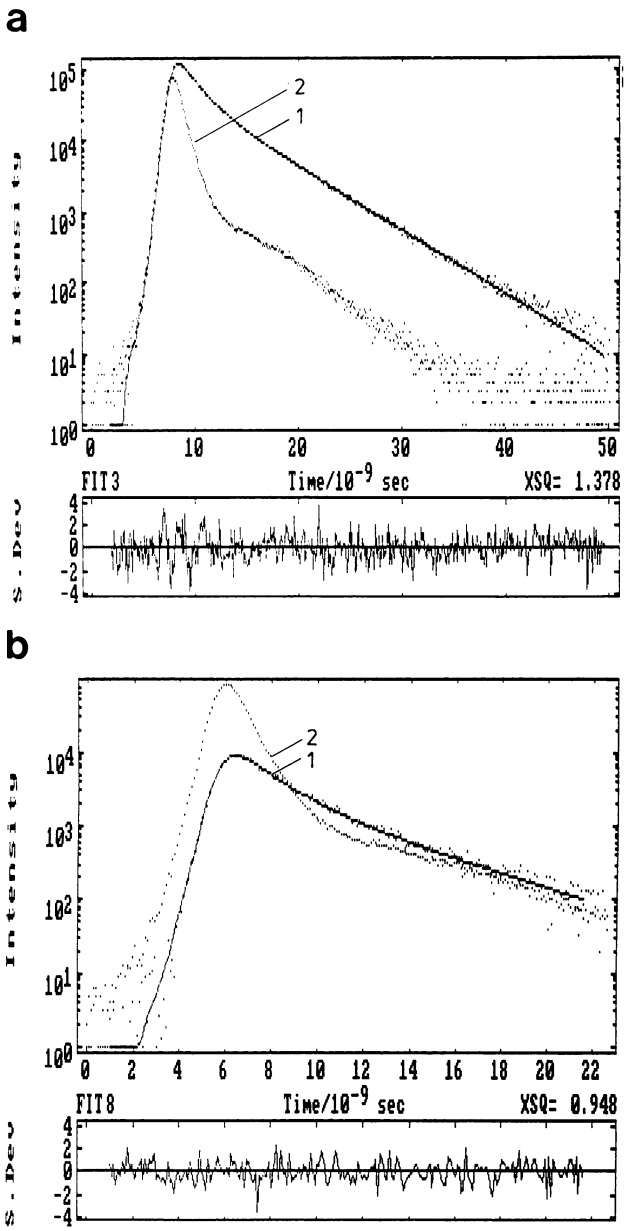

Fig. 6. The total fluorescence intensity decay (**a**) and the anisotropy fluorescence decay (**b**) of the EGF receptor external domain (**1**) and the fitted decay (———). The corresponding residuals are shown below. The total intensity was fitted by a triple-exponential decay and the decay of the difference using a single rotational correlation time as discussed in Materials and methods. The excitation was at 295 nm and the lamp profile measured at 333 nm (**2**).

the purified protein with and without human EGF was analysed by sucrose gradient centrifugation. When albumin and immunoglobulin IgG were run on a 5–25% sucrose density gradient and the fractions analysed by SDS–PAGE, albumin was located around fraction 12 and immunoglobulin located around fraction 18. When the external domain of the EGF receptor before and after addition of ligand was applied to an identical sucrose gradient saturated in human EGF, the receptor was located in both cases around fraction 14 (see Figure 7). In addition to SDS–PAGE analysis, fractions from the gradient were also analysed (after dialysis to remove excess EGF) for [I^{125}]EGF binding using a PEG precipitation assay. Ligand-binding activity was associated

Table III. The decays of the total fluorescence intensity and fluorescence anisotropy

Sample	Total fluorescence intensity decay lifetimes							Fluorescence anisotropy decays				
	b_1	b_2	b_3	t_1	t_2	t_3	χ^2	r_0	b	r_∞	t_r	χ^2
Human EGF	19.3	68.1	12.6	0.66	2.62	5.28	1.27	0.096	0.09	0.006	2.00	1.16
Receptor	26.0	39.6	34.4	0.58	2.16	4.9	1.38	0.072	0.029	0.043	6.24	0.95
Receptor:hEGF (1:1)[a]	20.2	62.9	16.9	1.14	3.48	7.25	1.37	0.109	0.051	0.058	2.70	1.34
Receptor:hEGF (1:2)	17.9	52.9	29.1	0.62	2.47	5.48	1.87	0.092	0.045	0.047	2.23	0.96
Receptor:mEGF (1:1)	19.1	50.2	30.7	0.63	2.51	5.70	1.55	0.103	0.045	0.057	2.53	0.98
Receptor:α-TGF (1:1)	22.4	45.7	31.9	0.74	2.63	5.66	1.45	0.107	0.044	0.063	2.44	1.06

[a]Measured without using the filter UG11.
The three lifetimes t_i and their respective amplitudes b_i were calculated as discussed in Materials and methods. χ^2 (chi squared) was used to monitor the quality of fit to the observed data. The limiting anisotropies r_0 and r_∞ and the correlation time t_r and amplitude b were also calculated as discussed in Materials and methods.

with fractions 12–16 and only non-specific binding was noted in the fractions from other parts of the gradient. Using this method, no detectable aggregation or dimerization was evident; thus the differences seen in the CD spectra implicate a real conformational change in a monomeric receptor.

Discussion

This study reports the properties of the ectodomain of the human EGF receptor secreted by insect cells using a suitably engineered baculovirus vector. The recombinant virus expressed ~8 mg of receptor per 5×10^9 cells, which is higher than the expression level achieved for the external domain of the insulin receptor using the bovine papilloma virus in NIH3T3 cells (Whittaker and Okamoto, 1988). The external domain produced was correctly folded and secreted by Sf9 cells into the culture medium. Using a simple three-step purification protocol, 1 mg of purified receptor was routinely recovered from 1 l of conditioned medium. The protein was soluble in the absence of detergents at the concentrations used in this study and had an affinity for EGF comparable to that of detergent solubilized EGF receptor from A431 cells.

The quantities of the EGF receptor ectodomain produced here have allowed the application of biophysical techniques to the study of a growth factor receptor for the first time. CD spectroscopy was used to assess the secondary structural composition of the EGF receptor ectodomain. The far-UV spectrum displays large negative CD bands at 208 and 217 nm (6000 mean residue weight theta) and is characteristic of a globular protein containing α-helix and β-sheet (Manavalan and Curtiss Johnson, 1983). There is overall agreement with the model suggested by Bajaj *et al.* (1987), although more β-sheet is found. This may result from contributions to the CD spectrum from glycosylation of the receptor domain. The exact proportion of carbohydrate, more specifically of *N*-acetylglucosamine, in the insect-derived human EGF receptor remains quantitatively undetermined (Greenfield *et al.*, 1988). Such sugar moieties have been shown to contribute a negative ellipticity below 210 nm (Bush *et al.*, 1980, 1982), and this may partially account for the relatively small positive peak seen at 189 nm which is usually much larger for more helical proteins (Brahms and Brahms, 1980). In some instances the

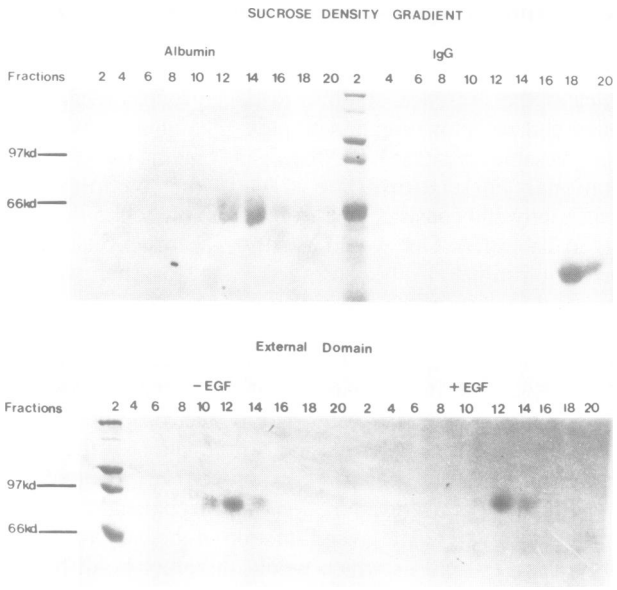

Fig. 7. Sucrose density gradient centrifugation of the uncomplexed and complexed receptor. Fractions (200 μl) were aliquoted from the gradients and run on SDS–PAGE under reducing conditions and silver stained. For comparison, BSA at 1 mg/ml and monoclonal antibody R1 also at 1 mg/ml were used as standards as described in Materials and methods.

contribution of carbohydrate can be estimated from spectra of model oligosaccharides that have structures similar to those found in glycoproteins (Bush *et al.*, 1981). However, possible interference of sugar moieties is not taken into account by the CONTIN program and may have led to a decreased helix/sheet ratio in this case.

In the near-UV spectrum of the receptor domain the main band originates from tyrosyl side chains. In addition there is fine structure between 250 and 270 nm usually associated with phenylalanine. The weaker extrema at 290 and 300 nm may be due to tryptophan constrained in an asymmetric environment. In view of the large cystine or cysteine content of the external domain, there may be an underlying contribution from the disulphide bonds, its magnitude, sign and wavelength position depending on their geometry. The complexity of the near-UV spectrum renders interpretation

in terms of individual components impossible (Kahn, 1979).

Although CD spectroscopy has limitations in the determination of absolute amounts of secondary structure, it is particularly suitable as a sensitive probe of structural differences or changes. For this study two different growth factors (human EGF and TGF-α) with only 20% sequence identity but with similar binding affinities for the EGF receptor were used. Their spectra exhibit pronounced differences in both the near- and far-UV (data not shown) and will be discussed elsewhere (manuscript in preparation). Here they were used to allow a difference analysis of the spectra of the receptor in the presence and absence of ligand. Similar studies on the insulin receptor external domain using EGF as the negative control remarkably showed the non-specific nature of the aggregation observed (Johnson *et al.*, 1988), but this was not the case for the EGF receptor external domain.

The differences (see Figure 4) observed in the far-UV between these two states of the receptor are small, but are they significant? Significance is ascertained by reproducibility. In principle, two proteins can form a complex and produce CD spectral effects without undergoing a conformational change, simply due to mutual perturbation of transitions in the two partner molecules brought into close proximity (Bayley, 1973). The effects observed here, however, are spread over the whole wavelength range and, more importantly, they are essentially the same for the complexes with both ligands, although the CD spectra of these ligands in the uncomplexed state are substantially different. These observations support the interpretation that the differences shown in Figures 4 and 5 are caused by a conformational change. It cannot, of course, be decided whether the conformational change occurs in receptor or ligand. Indeed it is likely that changes will occur in both molecules. In addition, the change in absolute ellipticity observed in the near-UV (see Figure 5) may also provide a more sensitive conformational probe to changes on ligand binding. A more systematic analysis using several genetically engineered EGF mutants to probe the observed spectral changes in more detail is now underway.

For the steady-state fluorescence data the largest changes occur to the growth factors themselves since their fluorescence emission is shifted towards shorter wavelengths. This indicates that the tryptophan side chains of the ligands are brought into an environment with a greater extent of shielding from solvent. Thus the steady-state λ_{max} of the receptor−ligand complex is the same as that of the uncomplexed receptor.

A comparison of the fluorescence spectra of free receptor with those of the binary complexes shows differences in the following parameters: the ratio of the fluorescence intensity and absorbance, i.e. the quantum yield, the pre-exponential weighting factors, the lifetimes from the total fluorescence intensity, and the rotational correlation times calculated from fluorescence anisotropy decay. As these differences in tryptophan fluorescence are the same for all three ligands, using α-TGF as a control (since it has no tryptophan side chains) it can be concluded that the observed effects are due to changes within the receptor. The increase in quantum yield could be due to the removal of a quenching group from a tryptophan environment. A specific side chain residue present on mouse EGF but not on the other two molecules may explain why there is no increase on binding of mouse EGF. The most interesting point is the reduction of the rotational correlation time, indicating an increased mobility of the receptor when complexed with ligand. Since the observed effect is the average of six tryptophans present in the receptor, it is likely that this is due to a change in the overall receptor conformation, but it could also result from a single tryptophan which dominates the fluorescence spectrum after ligand addition. The changes observed by CD and fluorescence measurements would have their simplest explanation in a specific ligand-induced conformational change in the receptor and ligand on complex formation. The centrifugation data indicate that no dimerization occurs in the receptor secreted from insect cells. However, dimerization has been shown to take place in the full-length receptor (Boni-Schnetzler and Pilch, 1987; Yarden and Schlessinger, 1987a,b) and suggest that a membrane anchor or cytoplasmic domain may be required for this phenomena to occur. An interesting comparison could be made with respect to the aggregation state of the truncated EGF receptor secreted by A431 cells (Ullrich *et al.*, 1984). However, it has not yet been established whether this molecule is able to dimerize.

Future studies will use site-directed mutagenesis to probe regions such as the L_2 domain, which comprises residues 333−460 (Bajaj *et al.*, 1987), and has been implicated to form a major part of the EGF binding site (Lax *et al.*, 1989). Such mutants can then be overexpressed, using the system discussed, in sufficient quantities to allow similar biophysical measurements.

Materials and methods

Materials

The IPL-41 cell culture medium was purchased from JR Scientific. Fetal calf serum was from Gibco. All FPLC apparatus and columns were from Pharmacia. The Aquacide II was from Calbiochem. The recombinant polypeptides human EGF and TGF-α were a generous gift from Dr G.Carlos-Nascimento, Chiron Corp., USA and Dr H.Gregory, ICI. Mouse EGF was purified according to Savage *et al.* (1972). The Sf9 insect cells and the plasmid pAc401 was donated by Max Summers, Texas A&M.

Cloning constructs

The cloning of the cDNA encoding for the external domain of the EGF receptor into the baculoviral vector pAc 401 required a series of cloning strategies. Basically, the cDNA of the receptor in pUc18-EGFR was digested in two stages and the *Sst*I−*Bam*HI fragment (−43 to 1350) was ligated to the *Xho*II fragment (1350−2115), in a mp13 vector which was labelled A6. An oligonucleotide, ATCCGGAACGGCCGCTCGAG, was then inserted into Tg130 and this resulting vector A4 was used to accept an *Xma*III−*Sal*I digest from A6, resulting in A8. A *Kpn* site was then inserted into pAc 401 enabling the insertion of an *Eco*RV−*Kpn*I digest from A8, encoding for the external domain to be ligated resulting in the final plasmid construct A13. This plasmid has the full polyhedrin promoter together with the first two amino acids of the polyhedrin gene (which are identical to those of the EGFR), followed by the coding sequence of the human receptor up to the penultimate amino acid before the transmembrane region. At this point the 620th amino acid proline has been substituted for leucine and the serine at position 621 becomes a stop signal.

Tissue culture

Cloned *Spodoptera frugiperda* Sf9 cells were grown at 27°C in suspension culture in 1 l of IPL-41 medium supplemented with 10% FCS using the Techne MCS 104L biological stirrer. The IPL-41 medium was supplied by JR Scientific and prepared according to Weiss and Vaughn (1986). The cells were seeded at a density of 1×10^5/ml and grown to 1×10^6/ml before infecting them with the recombinant virus at a multiplicity of 10 p.f.u./cell. Four days post-infection the suspension culture was centrifuged using a Sorval GS3 rotor at 8000 r.p.m. for 30 min at 4°C and the cell pellet discarded.

Purification of the external domain of the EGF receptor

The supernatant from the equivalent of 10^9 cells was passed through a 10 ml lentil lectin column, pH 7.5, at a flow rate of 1−1.5 ml/min. The column was then washed with 10 column volumes of 5 mM Tris−HCl, 0.5% Triton X-100, pH 7.5, before eluting with 0.2 M methyl α-D-mannopyranoside (Sigma M6882) in the same buffer. The eluted material was then dialysed against 20 mM Tris−HCl, pH 8.0, in 10% glycerol before it was applied to a MonoQ 10/10 column (Pharmacia). The bound material was then eluted with a NaCl gradient (0−100 mM) and the fractions containing EGF binding activity were pooled. This material was then concentrated using Aquacide II (Calbiochem) and then loaded on a superose 12 column, previously calibrated with blue dextran 2000, thyroglobulin, ferritin, albumin, catalase and acetone. The column was run in 20 mM Tris−HCl, 150 mM NaCl at a rate of 0.2 ml/min and again fractions with EGF binding activity were pooled and concentrated by speed vacuum centrifugation The end product was finally dialysed against phosphate buffer 50 mM, pH 8.0, ready for CD and fluorescence analysis.

Purification of the polypeptide growth factors

Recombinant human EGF and human α-TGF were finally purified by HPLC (Applied Biosystems Model 151A). Approximately 1 mg of each polypeptide was individually loaded onto a 250 × 7 mm C8 RF300 column (Brown Lee Laboratory) in 0.08% trifluoroacetic acid (TFA) and eluted with an acetonitrile/0.08% TFA gradient 0−63%, over 40 min at a flow rate of 3.6 ml/min. Fractions were collected every 30 s and the absorbance at 206 nm was measured. The authenticity of the polypeptide was then confirmed by amino acid analysis (Applied Biosystems Model 420), and by its competition for I^{125}-labelled EGF binding to A431 cells.

Detection of the external domain and Scatchard analysis

For Scatchard analysis, 30 μl of the purified material was added to 5 μl (120 pg) of [I^{125}]EGF and the reaction volume made up to 40 μl with increasing concentrations of unlabelled EGF (2.5−100 ng). The reaction was incubated for 1 h at 24°C, followed by the addition at 4°C of 10 μl of EGF-R1 antibody (0.2 mg/ml). After 10 min, 10 μl of cold 0.24% γ-globulin and 50 μl of 25% polyethylene glycol (PEG 6000) were added at 4°C and incubated for a further 20 min. The insoluble material was pelleted and washed in 25% PEG and the γ disintegrations counted. The data were corrected for non-specific binding (always < 10% total counts) and analysed by the method of Scatchard (1949).

Preparation of samples for amino acid analysis and N-terminal sequencing

The polypeptides eluted from the Applied Biosystems peptide purification system (Model 151A) at ~30% acetonitrile/0.08% TFA. These fractions were freeze dried and then vapour hydrolysed with 6 M HCl/1% phenol under argon at 165°C for 60 min. The sample was then taken up in 20 μl of a 250 p.p.m. solution of EDTA in HPLC purity water and applied on a sample slide and delivered to the Applied Biosystems Model 420 A Derivatizer-Analyser equipped with an Applied Biosystems Model 920A Data Analysis Modulator. For N-terminal amino acid sequencing, ~50 μg of purified protein was incubated in 0.05% SDS at 37°C for 1 h and then dialysed overnight against 0.1% TFA. This material was then added to the Applied Biosystems Model 471A gas-phase sequencer with a 120A on-line phenylthiohydantoin analyser, and the protein sequentially Edman degraded from the amino terminus.

Sucrose density gradients

Four 5−25% sucrose gradients (4 μl) were made (tubes a−d) and equilibrated at 4°C for 1 h. Purified external domain (100 μl 0.1 mg/ml) ± human EGF (100 nM) were added to their respective tubes. For the ligand−receptor complex, the external domain was equilibrated for 10 min at 20°C with EGF prior to centrifugation and to maintain the complex; the sucrose gradient was also equilibrated throughout with human EGF (100 nm). The protein standards used were 100 μl of BSA (1 mg/ml), and monoclonal antibody R1 (1 mg/ml). The tubes were then centrifuged in a SW60Ti rotor at 55 000 r.p.m. for 19.5 h at 4°C with slow acceleration and deceleration. Following centrifugation the contents of each tube were aliquoted into 200 μl fractions by withdrawing the sucrose solution (67 μl at a time) from the top of each gradient. Each fraction was then analysed by SDS−PAGE for protein and by [I^{125}]EGF binding for ligand binding activity.

Circular dichroism measurements

CD measurements were recorded on an AVIV Model 62DS CD spectrometer prototype, calibrated with *d*-10-camphosulphonic acid ($E_{290.5}$ = 2.31 mol/cm). The spectral bandwidth was 1.5 nm and measurements were conducted at room temperature. The time constants ranged between 1 and 8 s and the cell path-lengths between 0.1 and 5 mm. The concentration of the growth factor polypeptides was determined photometrically using the following adsorption coefficients based on quantitative amino acid analysis. Human EGF $A^{g/l;\ 1\ cm}_{280\ nm}$ = 3.1, human α-TGF $A^{g/l;\ 1\ cm}_{280\ nm}$ = 0.35, mouse EGF $A^{g/l;\ 1\ cm}_{280\ nm}$ = 3.1. The receptor concentration was determined photometrically using an absorption coefficient $A^{g/l;\ 1\ cm}_{280\ nm}$ = 0.74 and from the Bradford assay method measured at 595 nm, using γ-globulin as a standard. The solvent used was 50 mM phosphate buffer, pH 8.0. The receptor and both growth factors were measured separately. For the measurement of the binary complexes, aliquots of the receptor stock solution were incubated for 15 min with equimolar amounts of each ligand to a constant final volume. Three scans were performed and averaged for each sample. CD spectra were analysed in terms of main-chain conformation with the CONTIN program, which is based on a least-squares fitting routine using the CD spectra of 16 reference proteins (Provencher and Glockner, 1981).

Steady state, lifetime and anisotropy fluorescence decay measurements

The steady-state fluorescence spectra were recorded on a Spex Fluorolog 211 fluorimeter with a bandwidth of 2.7 nm (excitation monochromator) and 2.2 nm (emission monochromator). They were recorded as a ratio of emission to lamp reference and are corrected for spectral sensivity of the monochromator−photomultiplier system. The fluorescence lifetimes were measured with an Edinburgh Instruments lifetime spectrometer, model 199. The bandwidth of the excitation monochromator was 18 nm and the full width half maximum (FWHM) of the lamp pulse was ~1.35 ns. To eliminate stray light, a Schott filter WG320 was used, in addition a Schott filter UG 11 was also used to cut off wavelengths >390 nm where some contamination from plastics was evident. The instrument response function was recorded with a Ludox (DuPont) suspension using incident light at the wavelength of maximal fluorescence emission since the timing and shape of the photomultiplier output is more wavelength dependent than the hydrogen flashlamp. Data was accumulated to 80 000 counts in the peak channel and the decays were deconvoluted using a non-linear least-squares fitting program supplied by Edinburgh Instruments. The total fluorescence intensity decays observed were fitted to a sum of exponential terms of the form $\Sigma\ a_i \exp(-\lambda t/t_i)$ where t_i is the *i*th lifetime of the fluorophore's excited state. Additional lifetimes were introduced if it improved the quality of fit to the measured decays. The relative amplitudes for each lifetime were calculated from $b_i = a_i\ t_i / \Sigma\ a_j\ t_j$. The fluorescence anisotropy decay obeys $r(t) = b\ \exp\ (-t/t_r + r_\infty)$, and also $r_0 = b + r_\infty$ where r_0 is the limiting anisotropy in the absence of rotational diffusion (i.e. $t = 0$) and r_∞ is the limiting anisotropy as $t \rightarrow \infty$. T_r is the average rotational correlation time of the sample, and *b* the amplitude of the decay. The same protein samples used for CD measurements were used but were diluted to give an absorbance of 0.1 at 280 nm in a 1 cm cell and measured at room temperature.

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