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# Recognition of an antiparallel β-sheet structure of human epidermal growth factor by its receptor

## Site-directed mutagenesis studies of Ala-30 and Asn-32

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The Ala-30 and Asn-32 residues involved in the major antiparallel β-sheet structure of human epidermal growth factor (hEGF) were substituted with various amino acid residues, and the receptor-binding affinities of the nine variant hEGFs were determined by the use of human KB cells. The Ala-30→Arg, Ala-30→His and Ala-30→Phe substitutions drastically reduced the binding affinity, suggesting that the side chain in position 30 of Ala-30 of hEGF is required to be small for the receptor binding. The Asn-32→Asp substitution significantly reduced the binding affinity, while the Asn-32→His variant could bind to the receptor as well as to the wild-type hEGF. Therefore, it seems to be important for receptor binding that the side chain in position 32 does not have a negative charge but does have an NH group. Thus, we propose that, in the ligand–receptor complex, the receptor recognizes, on one side of the antiparallel β-sheet structure of hEGF, a wider contact area than previously suggested.

Epidermal growth factor; Site-directed mutagenesis; Human epidermal growth factor receptor; Protein engineering

## 1. INTRODUCTION

Epidermal growth factor (EGF) promotes cell proliferation through multi-step signal transduction triggered by binding of EGF to its receptor on the surface of target cells [1–3]. However, the mechanism of the receptor binding of EGF has not been fully elucidated at the molecular structure level. So far, several amino acid residues have been found to be important for the receptor binding, for example Arg-41 and Leu-47 [4–10].

Chemically synthesized peptides containing residues 20–31 of EGF bind, though weakly, to the receptor, suggesting the importance of this region for the receptor binding [11,12]. By nuclear magnetic resonance (NMR) spectroscopy, it has been shown that this segment (residues 20–31) of EGF is involved in the major antiparallel β-sheet structure [13–17]. In human EGF (hEGF), residues 18–23 and 28–33 form a β-sheet structure and residues 24–27 form a β-turn, where the side chains of residues 19, 21, 23–28, 30 and 32 are exposed on the protein surface (Fig. 1) [13,14]. These exposed residues have the potential of interacting directly with the receptor. Among the residues on the β-strand involving residues 19–23, Ile-23 has been shown to be important for the receptor binding [18,19]. Further, a combination of mutagenesis and NMR studies have shown that the side chain of Ile-23 is directly recognized by the receptor [19].

In contrast, the role of the other β-strand, involving residues 28–33, in the receptor binding has not been elucidated, because no mutagenesis study has been made of Ala-30 and Asn-32. In the present study, we substituted Ala-30 and Asn-32 of hEGF and examined their involvement in the receptor binding.

*Abbreviations:* EGF, epidermal growth factor; hEGF, human EGF; [$^{125}$I]mEGF, $^{125}$I-labeled mouse EGF; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 2D NOESY, two-dimensional nuclear Overhauser effect spectroscopy.

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## 2. MATERIALS AND METHODS

### 2.1. *Preparation of hEGF variants*

Mutations were introduced into the synthetic gene for hEGF on plasmid pTA1522 [20] using a Muta-Gene kit (Bio-Rad) [19]. *Escherichia coli* strain YK537 was used as a host for the pTA1522 derivatives bearing hEGF variant genes. Biosynthesis and purification of variant proteins were carried out as described [19].

### 2.2. *Assay of hEGF variants*

Concentrations of the wild-type and variant hEGFs were deter-

mined by amino acid composition analysis with an L-8500 system (Hitachi, Tokyo, Japan). The receptor binding affinities of hEGF variants were determined by radio-receptor competition assay; inhibition of the binding of $^{125}$I-labeled mouse EGF ([$^{125}$I]-mEGF) (Amersham, specific activity: 3.7 MBq/$\mu$g) to human KB cells (Dainippon Pharmaceutical Co., Osaka, Japan) was measured as described [21].

2.3. *NMR measurements of hEGF variants*

The 500-MHz two-dimensional proton NMR spectra of the wild-type hEGF, the Ala-30→Phe variant and the Asn-32→Asp variant (1.5 mM, pH 2.5 and 25°C) were measured by two-dimensional nuclear Overhauser effect spectroscopy (2D NOESY) [22] (mixing time = 80 or 100 ms) as described [19].

## 3. RESULTS AND DISCUSSION

By site-directed mutagenesis, Ala-30 was replaced by Arg, Gly, His, Phe and Val, and Asn-32 was replaced by Asp, His, Phe and Val. These variant hEGFs were obtained with nearly the same yield as that of the wild-type hEGF. The affinities of the purified wild-type and variant hEGFs to human KB cells were determined in competition with [$^{125}$I]mEGF (Fig. 2). The $IC_{50}$ values of hEGFs were estimated from the concentrations of the proteins at which the amount of cell-bound [$^{125}$I]mEGF was reduced to 50% of the maximum level. Relative binding affinities were obtained from $IC_{50}$ (wild-type)/$IC_{50}$ (variant) (Fig. 3).

Substitution of Ala-30 with Val, a slightly larger residue than Ala, slightly affected the binding affinity (Fig. 3). By contrast, substitutions with much larger residues, Phe and His, drastically reduced the binding affinity of hEGF down to 2.8 and 3.3%, respectively. To examine effects of the Ala-30→Phe substitution on the tertiary structure of hEGF, we measured 2D NOESY spectrum of the Ala-30→Phe variant (Fig. 4B). As sensitive probes for the tertiary structure of hEGF, we focused our attention on the nuclear Overhauser effects (NOEs) of Tyr-22 and Tyr-29 in a hydrophobic core [13,14], as we did previously for the Ile-23→Asp variant of hEGF [19]. The inter-residue NOEs due to the formation of the secondary and tertiary structures as well as the intra-residue NOEs were observed for the variant hEGF to the same extent as those for the wild-type hEGF (Fig. 4A). For example, the NOE cross-peak for the pair of the ring protons of Tyr-22 and the $\alpha$-proton of Tyr-29 (cross-peak a, Fig. 4B), and the NOE cross- peak for the $\alpha$-protons of Tyr-22 and Tyr-29 (data not shown) were observed in both spectra, indicating that the substitution did not affect the antiparallel $\beta$-sheet structure of hEGF. Further, for both proteins, the NOE between the ring protons of Tyr-29 and the $\beta,\beta'$-protons of Tyr-13 were observed (cross-peak b, Fig. 4A and B), indicating that the hydrophobic core structure involving Tyr-13, Tyr-22 and Tyr-29 [13,14] was hardly affected by the Ala-30→Phe substitution. From these results we concluded that the tertiary structure of the Ala-30→Phe variant is practically the same as that of the wild-type hEGF. Therefore, the drastic loss of the affinity indicates the importance of the side chain of Ala-30 for the receptor binding. Further, substitution of Ala-30 with another large residue, Arg, remarkably reduced the affinity to 9.6%. These results indicate that the side chain of the residue 30 is required to be small. When Ala-30 was substituted with Gly, a smaller residue than Ala, the affinity was reduced to 31%, possibly because Gly was too small.

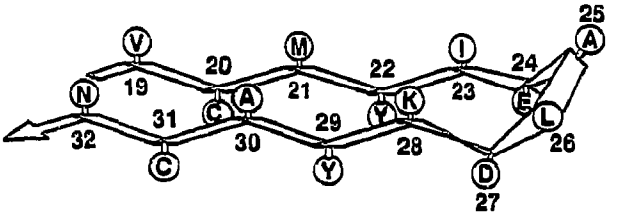


Fig. 1. Schematic view of the major antiparallel $\beta$-sheet structure in hEGF.

In the case of Ile-23, the side chain is strictly recognized by the receptor [19]; the Ile-23→Ala substitution reduced the affinity of hEGF down to 17% while the Ala-30→Val substitution reduced the affinity to 57%. Thus, the recognition of Ala-30 by the receptor is less strict than that of Ile-23. The position corresponding to Ala-30 of hEGF is occupied by a relatively small residue in other EGFs; Thr in mouse EGF, Val in rat EGF and Ala in Guinea-pig EGF [23–25], also indicating the requirement of a small residue in this position. The importance of hydrophobicity of the segment involving residues 19–29 has been proposed [26]. In contrast, the

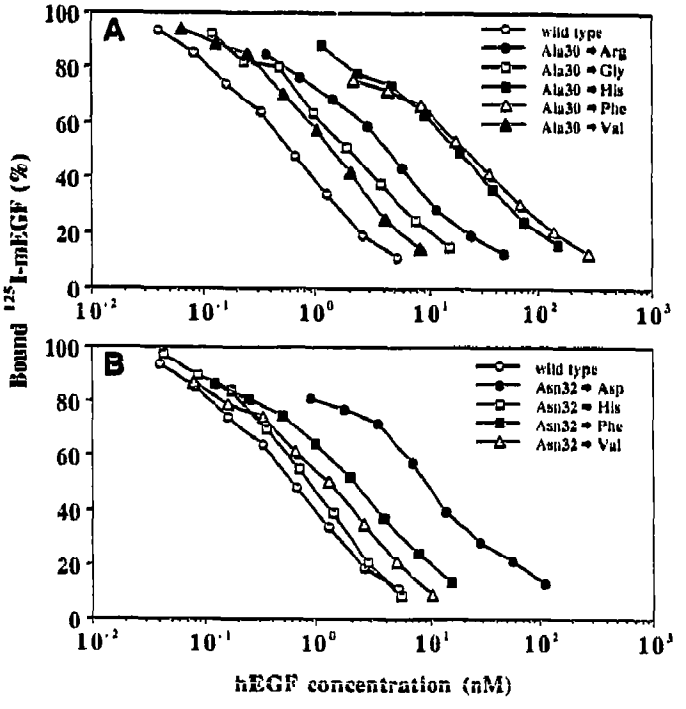


Fig. 2. Competition receptor binding of [$^{125}$I]-mEGF vs. the wild-type hEGF and variant hEGFs which have a mutation in position 30 (A) or 32 (B).

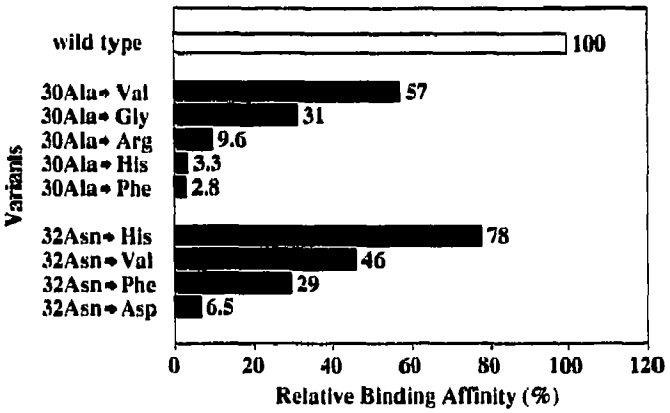


Fig. 3. Relative binding affinities of variant hEGFs. The number on the right side of the bar is the value of the relative binding affinity (%) compared to that of the wild-type hEGF.

hydrophobicity of the side chain in position 30 is not important for the receptor–ligand interactions (mouse EGF has a hydrophilic residue, Thr, in the position corresponding to Ala-30 [23] . Thus in position 30, a relatively small side chain is required for the receptor binding.

The Asn residue in position 32 is strictly conserved among all EGFs [23–25]. When this residue was substituted with Asp, the affinity of hEGF was remarkably reduced down to 6.5% (Fig. 3). As in the case of the Ala-30→Phe variant, we measured 2D NOESY spectrum of the Asn-32→Asp variant (Fig. 4C) and found that the spectrum was practically the same as that of the wild-type hEGF. Thus we concluded that the Asn-32→Asp substitution had a negligible effect on the tertiary structure of hEGF. Therefore the remarkable reduction in the affinity by the Asn-32→Asp substitution indicates the importance of Asn-32 for receptor binding. Replacement of Asn-32 with an uncharged residue, Val, less significantly reduced the affinity of hEGF. Probably, the reduction in the binding affinity upon the Asn→Asp substitution was caused by the introduction of a negatively charged side chain in position 32. Replacement of Asn-32 with Phe, a large and uncharged residue, reduced the affinity of hEGF more than the replacement with Val. The reduction in the affinity upon the Asn→Phe substitution suggests that a large side chain in position 32 is unfavorable for receptor binding. On the other hand, replacement by another large residue, His, did not reduce the binding affinity. Since the affinity measurement was performed at pH 7.4, it is likely that His was not positively charged and therefore had one NH group. This NH group may act in place of that of Asn. Thus, in position 32, a residue with an NH group in its side chain is favorable for receptor binding of hEGF.

In summary, we have shown here that the side chains of Ala-30 and Asn-32 are important for the binding of hEGF to the receptor. The residue in position 30 is

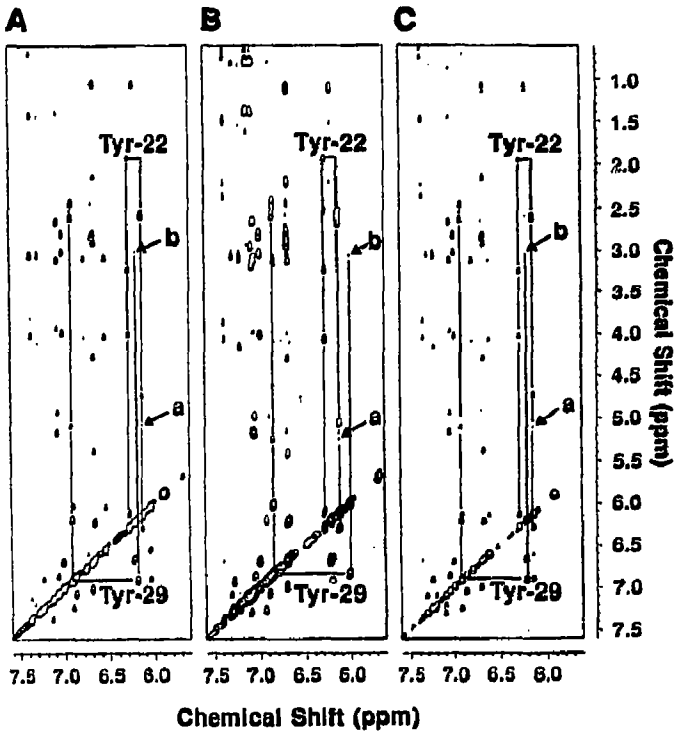


Fig. 4. 2D NOESY spectra (for pairs of the aromatic ring protons and aliphatic protons) of the wild-type hEGF (A), the Ala-30→Phe variant (B) and the Asn-32→Asp variant (C). The cross-peaks on the solid lines were due to the inter-residue NOEs (indicated with arrows and letters, a and b) and intra-residue-NOEs from the ring protons of Tyr-22 or Tyr-29.

required to be rather small, indicating that a large residue in this position disturbs the proper interaction between EGF and its receptor. A residue with an NH group in position 32 is favorable for receptor binding, suggesting the existence of a hydrogen bond acceptor in the EGF binding region on the receptor. Probably, the receptor directly binds with the $\beta$-strands of hEGF involving residues 30 and 32.

Previously, a peptide segment, residues 20–31, of hEGF had been found to bind weakly with the receptor [11]. In fact, Ile-23 in a $\beta$-strand, and Ala-25 and Leu-26 in a $\beta$-turn have been identified to be important for receptor binding [18,19,26]. In addition to these three residues, in the present study, we have identified two important residues, Ala-30 and Asn-32, in the other $\beta$-strand. Thus, a wide region of the major antiparallel $\beta$-sheet structure of hEGF, not simply the region of residues 23–26, is involved in the receptor binding. It should be noted here that the side chains of these five important residues are located all on the same side of the $\beta$-sheet structure (Fig. 1). Thus, we propose that the receptor binds with a wide contact area on the exposed side of the major antiparallel $\beta$-sheet structure of hEGF, including Ile-23 in a $\beta$-strand, and Ala-25 and Leu-26 in a $\beta$-turn, and Ala-30 and Asn-32 on the other $\beta$-strand.

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