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Receptor Cytoplasmic 영역에 의존하는 EGF의 고친화성 결합

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Dependence of High Affinity Binding of Epidermal Growth Factor on Receptor Cytoplasmic Domain

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ABSTRACT

Cell surface binding of epidermal growth factor (EGF) to EGF receptors was studied for a series of site-directed receptor mutants transfected into B82 mouse fibroblasts. Scatchard plots for truncation mutant receptors significantly lost nonlinearity for truncations below residue 1022. Transient plots of dissociation kinetics exhibited biphasic behavior for all receptor types, but the fraction of receptor in slow-dissociating form was reduced by an order of magnitude for the truncation mutants below residue 1022. Comparison of dissociation kinetics between control cells and cells treated with Triton X-100 revealed no significant variation for the slow-dissociating receptor form, but a noticeable variation was observed for the fast-dissociating receptor form when EGF receptors were truncated below residue 991. These results suggest that high affinity of EGF binding at cell surface depends on the EGF receptor cytoplasmic region.

INTRODUCTION

Binding of epidermal growth factor (EGF) to its receptor (EGFR) results in a cascade of events that lead to DNA synthesis and cell proliferation. Key initial events include activation of receptor tyrosine kinase activity (1, 2) and aggregation of receptors in coated pits for endocytosis (3). Quantitative studies of EGF binding have demonstrated behavior consistent with apparent multiple classes of effective binding affinity. These observations include nonlinear Scatchard plots for equilibrium binding experiments (4) and biphasic dissociation kinetics for transient dissociation experiments (5). Though the high-affinity class represents only a small portion, typically less than 10%, of cell surface EGFR, its biological function dominates receptor function (6). Thus, it is important to account for high-affinity ligand binding. The primary structures of both affinity classes are believed to be identical (7). Currently viable hypotheses for high affinity subclasses are receptor oligomerization (8) and coupling of receptors with mem-

brane-associated components (5). It is unclear whether either process might be related to aggregation of receptors in coated pits. Some evidence indicates that the high-affinity class is associated with the cell cytoskeleton (9, 10) and the cytoskeleton-associated receptor maintains ligand-binding and ligand-induced kinase activity (11, 12).

In this study, the series of EGFR mutants is exploited to investigate possible influence of the receptor cytoplasmic region on high-affinity cell surface binding. The results showed that both equilibrium binding and dissociation kinetics were dependent on the cytoplasmic domain structure. The dependence of receptor/cytoskeleton association and its relationship to binding affinity was explored using Triton X-100 extraction. The effects of this extraction on the dissociation rate constant for the fast dissociating form are noticeably different for EGFR truncations below residue 991, which demonstrate that the EGFR cytoplasmic domain plays a significant role in high-affinity cell surface binding of EGF, possibly by affecting the extent of receptor/cytoskeleton interaction.

MATERIALS AND METHODS

Cell Culture

B82 mouse L cells expressing wild-type recombinant human EGFR (WT), or C-terminal truncation mutants (△647, △973, △991, △1010 and △1022) have been previously described (13). B82 cells containing EGF receptors were grown in Dulbecco's Modified Eagle's medium (DME, Sigma) containing dialyzed 10% calf serum (Sigma) and methotrexate (5mM).

Radioiodination of EGF

Recombinant mouse EGF was iodinated with I^{125} (Dupont) using Iodo-beads (Pearce Chemical Co.) according to the manufacturer's recommendations. Free iodine was separated from the radiolabeled ligands by passing the mixture over a 0.8×20cm column (Kontes Chemical Co.) of Sephadex G-10 equilibrated with PBS saline buffer. The specific activity of ^{125}I-EGF was determined after precipitation of ligands using 1% of phosphotungstic acid containing 0.25% BSA and was generally between 100,000−200,000cpm/ng EGF.

Inhibition of Endocytosis by Phenylarsineoxide (PAO)

PAO (Sigma) solution is known to inhibit receptor-mediated endocytosis at 37℃ (14, 15). The preparation of the 0.1 mM PAO described elsewhere (16). Monolayer of B82 cells was rinsed once with HSP buffer, which contains 20mM Hepes, pH 7.4, 130mM NaCl, 5mM KCl, 0.5M $MgCl_2$, 1mM $CaCl_2$, 1mg/ml polyvinylpyrolidone. The rinsed B82 cells were incubated in the PAO solutions at 4℃ for 1h. The PAO treated cells were rinsed twice with the HSP buffer prior to adding prewarmed binding solution. Binding experiments were performed at 37℃ in a water bath.

Equilibrium Binding

With endocytosis of EGF receptors inhibited by 0.1 mM PAO solution, cells or isolated cytoskeletons were incubated in a 12 well plate (diameter 22mm) with D/H/B media containing from 0.1 to 50ng/ml of labelled EGF for 3 hours at 37℃, which was sufficient to obtain steady-state conditions for all EGF concentrations. At the end of incubation time, the cells or cytoskeletons were washed three times in HSP buffer and collected them in a tube to measure unbound ligands. Bound ligands were acid-stripped as described previously (13) and counted in a gamma counter.

Dissociation Analysis

B82 cells treated with 0.1 mM PAO solution were incubated with D/H/B media containing 20ng/ml of labelled EGF for 2 hours at 37℃. At the end of the incubation, the medium containing unbound free ligands was removed and the cells were washed twice with warm HSP buffer (37℃).

The washed cells were incubated with 1ml of warm D/H/B media containing 1.0 μg/ml of unlabeled EGF for a various time at 37℃. Unlabeled EGF was used to prevent the dissociated ^{125}I-EGF to rebind to cell surface. The surface-bound ligand concentration was determined as in equilibrium binding.

Cytoskeleton Isolation

Cytoskeletal elements of B82 cells were isolated by extraction for 10min with 0.5% Triton X-100 (TX) in 25mM Hepes (pH 7.4) and 1mM phenylmethylsulfonyl fluoride (PMSF) at 4℃. After removal of the Triton X-100-containing buffer, the residue-defined cytoskeletal elements were washed twice with the HSP buffer. This procedure was done in two different ways: (i) "TX extraction before" EGF binding, and (ii) "TX extraction after" EGF binding. In the former method, the TX extraction was done first, then the detergent extract was equilibrated with labelled EGF at 0℃. In the latter method, cells were equilibrated with labelled EGF at 0℃, followed by the TX extraction.

Scatchard plots were obtained using a range of EGF concentrations in both cases, as were dissociation kinetic plots using the equilibrated EGF binding as the initial condition for dissociation. Receptors bound by EGF under "TX extraction before" conditions are apparently those which are associated with cytoskeleton before EGF binding, while receptors bound by EGF under "TX extraction after" conditions include both these plus those for which cytoskeletal association is induced by EGF binding.

RESULTS

Equilibrium Surface Binding

The EGF receptor, consisting of 1186 amino acid residues, has several known functional domains: ligand-binding domain, transmembrane domain, tyrosine kinase domain, calcium modulation domain, and kinase regulatory domain (13, 17, 18, 19). However, receptor cytoplasmic domain responsible for high affinity EGF binding is still unknown. In order to elucidate the possible region, the series of receptors truncated at residues 1022, 1010, 991, 973, and 647 were prepared as diagrammed in Figure 1. As shown in Figure 2, Scatchard plot of equilibrium surface binding at 37℃ with wild-type EGFR (C1186) becomes nonlinear due to the biphasic affinity binding of EGF. The nonlinear Scatchard plot agree well to the results of quantitative analyses of a ternary complex model, in which the EGF/EGFR complex is postulated to couple with a stoi-

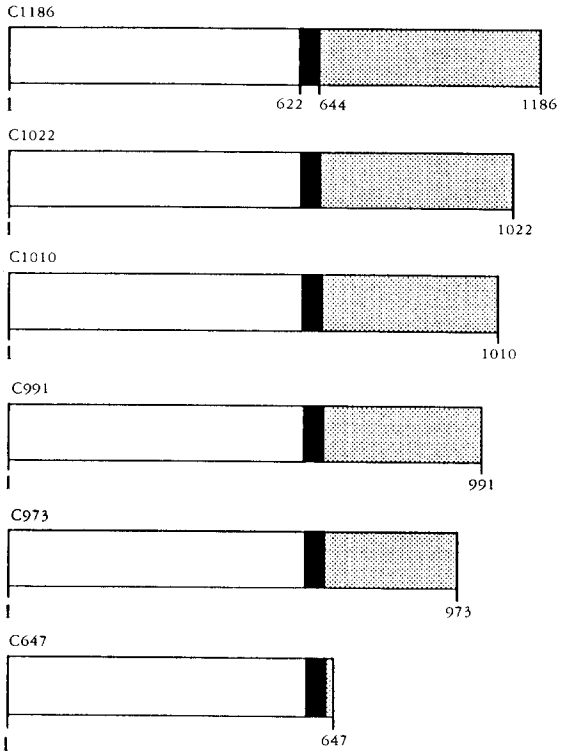

Figure.1. Mutant human epidermal growth factor receptors. Wild-type EGF receptor consists of 1186 amino acids containing extracellular domain (1−621), transmembrane domain (622−644), and cytoplasmic domain (645−1186). Mutant hEGF receptors are truncated at each indicated amino acid residue.

chiometrically-limiting membrane-associated component (5). Figure 2 also shows that the Scatchard plots for the series of EGFR truncation mutants became linear, indicating that the high-affinity fraction of EGF receptors diminishes significantly for truncation mutants below residue 1022. Although some nonlinearity is observed for the △991 receptor, it is relatively weak compared to the 1186 wild type forms.

Dissociation Kinetics

Transient plots of bound EGF during dissociation following ligand incubation are shown in Figure 3. As found previously by Mayo et al. (5), these plots

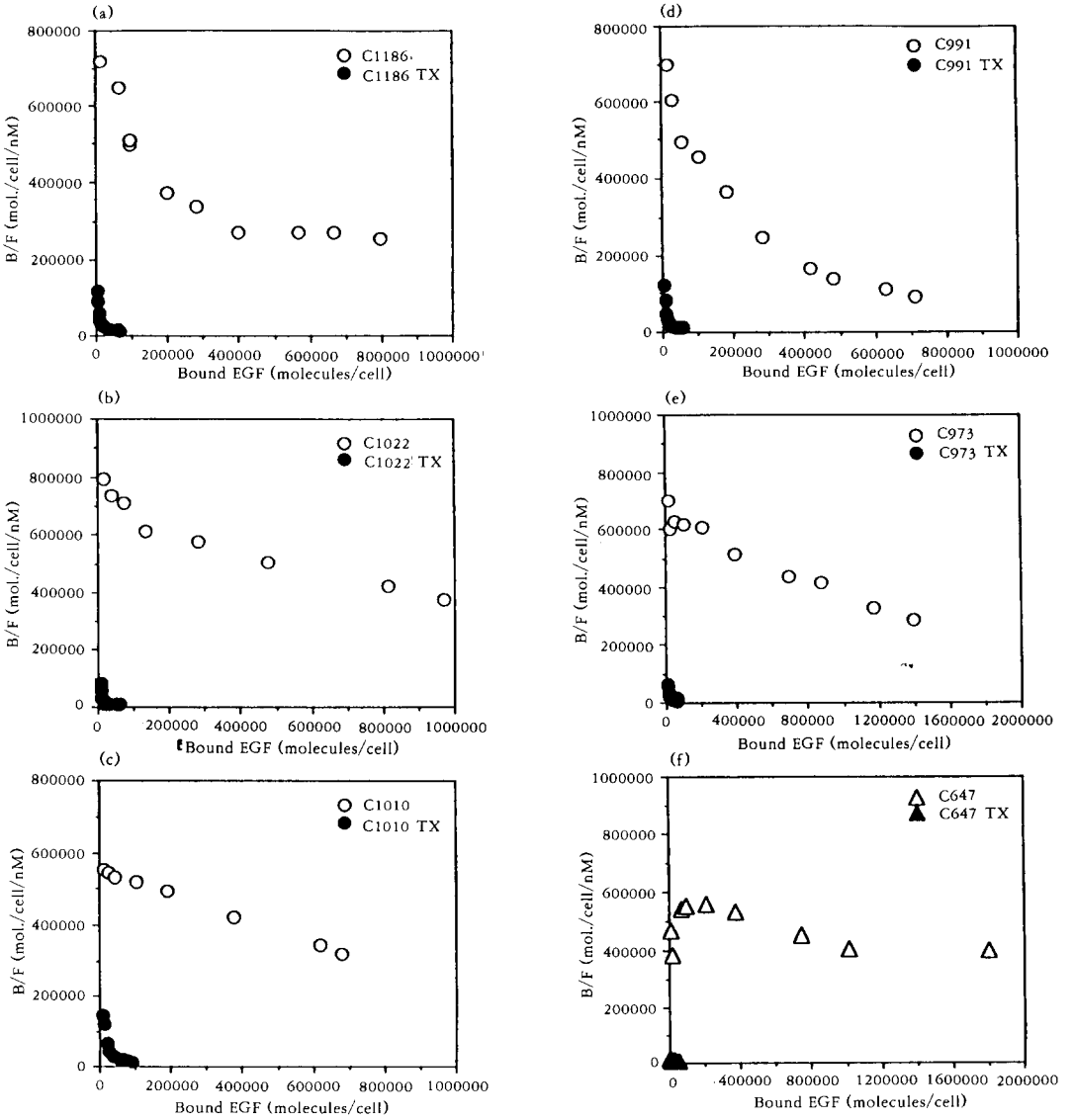

Figure.2. Scatchard analysis of ^{125}I-EGF binding to EGF receptors. Scatchard analysis was performed using ^{125}I-EGFs (0.3−50ng/ml) at 37℃ after PAO treatment. Closed circles represents experimental data obtained after Triton X-100 extraction before EGF incubation. B/F means Bound EGF molecules divided by Free (unbound) EGF molecules. Receptors are (a) C1186 wild type (b) C1022 (c) C1010 (d) C991 (e) C973 (f) C647.

exhibit biphasic behavior, with fast-dissociating (k_r) and slow-dissociating (k_u) components. The fraction of EGF/EGF-receptor complexes in the slow-dissociating class, at this incubation condition, is given by the intercept of the slow-dissociating curve with the ordinate axis. For the wild-type receptors (this fraction is about 2%) consistent with the range reported for the high-affinity receptor form from equilibrium binding data (4, 6). For the △1022, △1010, △991, △973, and △647 truncation mutants, this fraction falls to 0.4 %, 0.2 %, 0.5 %, 0.3 %, and 0.2 % respectively.

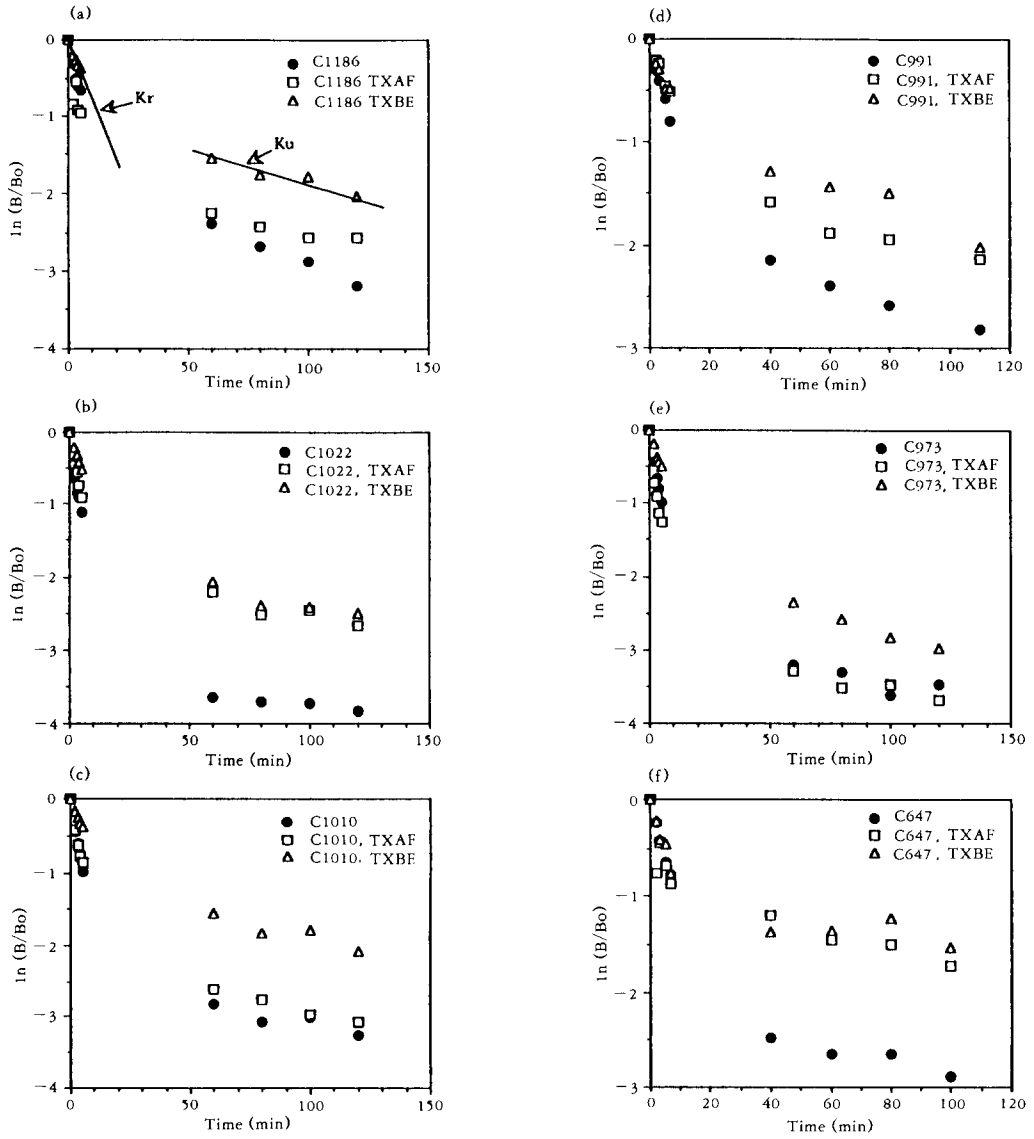

Figure.3. Dissociation rate of EGF from mutant EGF receptors. Surface bound ^{125}I-EGF was measured in the presence of 1 μg/ml of unlabelled EGF solution. Closed circles represents no detergent treatment. TXAF means Triton X-100 extraction after ^{125}I-EGF incubation and TXBE means Triton X-100 extraction before ^{125}I-EGF incubation. Receptors are (1) C1186 (b) C1022 (c) C1010 (d) C991 (e) C973 (f) C647.

Hence, the fraction of receptors in the slow-dissociating, presumably the high-affinity class, is reduced by a factor of roughly 5-to 20-fold for all the mutants. Most importantly, the fraction is substantially enough for the full-length receptors to provide nonlinear Scatchard plot behavior, while for the truncation mutants it is sufficiently small that Scatchard plots should appear linear.

Dissociation Kinetics with Triton X-100 Extraction

It has been suggested previously that cytoskeleton-associated EGFR represent the high-affinity class, as deduced from Scatchard analysis of EGF binding (11, 21). Thus, it is of interest to understand the structure interaction between EGFR and the cytoskeleton. Cells expressing mutant EGFR were incubated with PAO for 1hr at 4℃ in the absence of ^{125}I-EGF and extracted with 0.5% Triton X-100 for 10min at 4℃, which is sufficient to remove the soluble proteins and all phospholipids (11). After the extraction, the isolated cytoskeletons were incubated with ^{125}I-EGF, and EGF dissociation was determined at 37℃ in the presence of 1.0 μg/ml of unlabelled EGF, as described in Materials and Methods for the "TX extraction before" method. Figure 3 shows that the fast-and slow-dissociation sites are both distributed in Triton X-100 insoluble cytoskeletons, with the fraction in slow-dissociation class incresed over that in whole cells. For full-length receptors this fraction is about 30% to 60%, decreasing to the range 5% to 20% for the truncations. In isolated cytoskeletons, the fast dissociation rates decreased by about 50% as compared with the results of intact cells, but the slow dissociation rates were little affected, indicating that cytoskeleton-associated receptors predominantly contain slow-dissociating binding sites.

Analogous results were obtained for the "TX extraction after" method. Figure 4a shows rate constants corresponding to the fast-dissociating sites for the series of EGFR mutants. Notice the different patterns of detergent-extraction effects. For the △647 and △991 EGFR, the values of k_r are approximately equal for normal dissociation (i. e., without TX extraction), dissociation following TX extraction before binding, and dissociation following TX extraction after binding. WT EGFR and truncation mutants between △1022 and △1000 exhibit a progressive decrease in k_r values from normal, to "TX extraction after", to "TX extraction before". The △ 991 truncation mutant exhibits wild-type behavior for dissociation TX extraction. It is not clear whether the △ 973 truncation mutant fits into either of these patterns. Cytoskeleton association did not appear to affect the value of the slow-dissociation rate constant (Figure 4b), consistent with the possibility that this rate constant may correspond to the uncoupling of EGF/EGFR complexes from its ternary component (5).

DISCUSSION

Cytoplasmic sequences of the EGFR are known to be involved in EGF-induced signal generation and receptor internalization (13). Accordingly, it should be of interest to test the hypothesis that the cytoplasmic region of this receptor may also be important for regulation of ligand binding affinity.

Recently, it has been demonstrated that high-affinity EGFR are associated with the cell cytoskeleton (11, 12) and that EGF induces additional association of low-affinity binding sites to cytoskeleton components (10, 21).

In this work, equilibrium bindings and transient dissociation properties of a series of site-directed EGFR mutants are characterized in B82 cells. Scatchard analysis of equilibrium binding revealed that truncation below residue 1022 abolished noticeable presence of a high-affinity receptor subclass (Figure 2). Full-length and 1022 EGFR exhibited multiple affinity binding sites, consistent with the results of van Belzen et al. (22) in which the interaction of cytoskeleton with EGFR truncated at residue 1060 was found to be independent of receptor kinase activity.

Transient dissociation of EGF from EGFR is biphasic, similarly implying the existence of two classes of receptors: fast and slow-dissociating. Figure 3 shows that the fraction of receptors - dissociating form is diminished by an order of magnitude for truncations below residue 1022. Comparison of this observation with that for the equilibrium binding experiments supports the assumption that slow-dissociating binding sites are identical to high-affinity binding sites.

Especially, intriguing results were obtained for fast-component dissociation rate constants under normal conditions (without TX extraction) and following TX extraction before EGF binding or TX extraction after EGF binding (Figure 4a). For the WT EGFR, the values of k_r in both TX extraction procedures were significantly reduced from that found under nonextraction conditions, which indicates a stronger binding of EGF to the receptor when it is associated with cytoskeletal elements. For the △647 truncation mutant receptor, which is unable to associate with cytoskeleton, the values of k_r were not changed upon TX extraction in either method. Truncation mutants retaining a significant cytoplasmic domain, permitting some association with cytoskeletal elements, demonstrated intermediate behavior: generally, the value of k_r obtained following TX extraction performed after EGF binding was decreased somewhat from the normal value, while that following TX extraction performed before EGF binding was more substantially reduced. This suggests that receptor/cytoskeleton association induced by EGF/EGFR binding may be more strongly affected by the receptor cytoplasmic domain truncations than is the background, constitutive association. These results further support recent findings that EGF induces additional formation of EGF receptor and filamentous actin and unidentified proteins (10) and that EGF induces dimerization of soluble EGF receptor, which in turn resulted in increased ligand binding affinity (8).

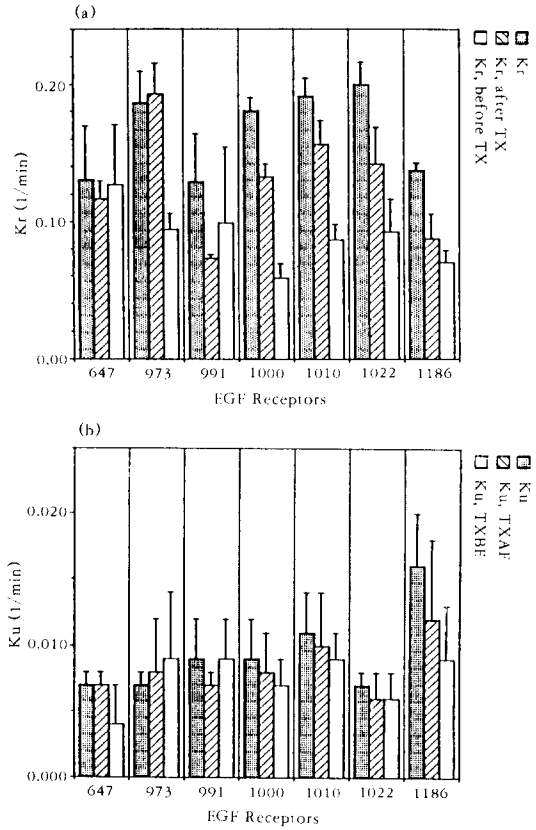

Figure.4. Summary of dissociation rates of EGFs from mutant EGF receptors. Slopes of (a) fast dissociating components, K_r, and (b) slow dissociating components, K_u, are expressed. Data include 95% error allowance from triplicate experiments.

In contrast, Figure 4b demonstrates that cytoskeleton association did not affect the value of the slow-dissociation rate constant. These data are consistent with the hypothesis that this rate constant may correspond to the uncoupling of EGF/EGFR complexes from its ternary component (5). At the same time, this figure shows that the value of k_r does not vary among the receptor truncation mutants. This would imply that the rate of ternary complex uncoupling is not affected by the cytoplasmic domain. Both equilibrium binding and transient dissociation in whole cells exhibited significantly altered properties for receptor truncation below residue 1022, indicating that the domain between 1022 and the car-

boxy terminus at residue 1186 is involved in ligand binding affinity by way of receptor-cytoskeleton association.

요 약

일부의 EGF receptor 에는 EGF 가 세포표면에서 receptor 와 결합할 때 보다 높은 친화력(high affinity) 을 보이고 있는데 그 이유를 설명하기 위해서 EGF receptor 의 cytoplasmic 영역을 절단하여 EGF 와의 친화력을 측정하였다. Scatchard plot 의 결과 1022 아미노산 이하로 절단된 receptor 는 high affinity 특성을 상실하였다. Triton X-100로 세포막을 제거하여 cytoskeleton 이 EGF receptor 의 구조에 미치는 영향을 조사한 결과 cytoskeleton 과 결합한 receptor 가 결합하지 않은 receptor 보다 EGF 에 대해서 더 높은 친화력을 보였다. 따라서 cytoskeleton 이 high affinity EGF receptor 를 형성하는데 영향을 미치고 receptor 와 cytoskeleton 의 가능한 결합부위는 1022−1186 아미노산 사이인 것 같다.

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