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Properties of the Proteolytically Generated Catalytic Domain (42 kDa Kinase) of Epidermal Growth Factor Receptor: Comparison with Holoenzyme

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Treatment of A431 cell membranes with trypsin or *Streptomyces griseus* proteinase results in degradation of the EGF-R and the concomitant generation of an active kinase with a molecular mass of 42 kDa (42 kDa kinase). To investigate the biochemical properties of the 42 kDa kinase, the EGF-R was immunoaffinity-purified from the A431 cell membranes and the kinase proteolytically generated. The proteolysis of EGF-R changes both the V_{max} and the Michaelis constants of substrates. These substrates determine the extent of the changes of the parameters. The 42 kDa kinase is less responsive to polyions as regulators of kinase activity and is less efficiently inhibited by genistein and tyrphostin. The experiments described here point to a role of the extracatalytic domains in determining the substrate specificity and regulation of kinase activity.

Key words: catalytic domain, EGF-R, protein kinase.

Binding of the EGF to its receptor stimulates EGF-R kinase activity and leads to internalization and down-regulation of the molecule (*1*). The fate of the internalized EGF-R remains unclear. Several reports show a proteolytic degradation of the internalized molecule with following generation of defined functional domains (*2, 3*). Evidence has been presented indicating that internalized receptor may be functionally active and able to generate intracellular signals (*4*). Tryptic digestion has allowed the recovery of a 42 kDa fragment of the EGF-R corresponding to its tyrosine kinase domain (*5*). Comparison of the properties of the 42 kDa kinase and EGF-R is interesting since the limited proteolysis converts the receptor into a constitutively activated kinase. This kinase could prolong the response of EGF-R on the transient stimulation by EGF (*6*).

In this study we have generated the 42 kDa kinase by limited proteolysis of the EGF-R and utilized it for kinetic studies. Based on comparison of the kinetic parameters of the 42 kDa kinase and the EGF-R, we conclude that the deletion of the ligand-binding domain and the autophosphorylation sites of the holoenzyme results in different properties.

EXPERIMENTAL PROCEDURES

Materials—Mouse EGF and immobilized anti EGF-R Ig 528 were obtained from Dr. W. Weber (Institut für Physiologische Chemie Hamburg). Trypsin (sequencing grade), *Streptomyces griseus* proteinase, BSA, poly(Glu^4, Tyr^1) (20-50 kDa), poly(Glu^6, Ala^3, Tyr^1) (20-50 kDa), poly(Lys) (1-4 kDa), α-casein and all other fine chemicals were from Sigma. Proteinase inhibitors and histone H2B were obtained from Boehringer Mannheim. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham and [^{14}C]FSBA (47.6 mCi/mmol) from DuPont. Autoradiography was performed using Kodak X-OMAT AR film.

Purification of EGF Receptor—Highly purified human EGF-R was isolated from A431 epidermoid carcinoma cells by immunoaffinity chromatography, according to the procedure described by Weber *et al.* (*7*). Briefly, detergent cell membrane extracts were passed through a column containing anti-EGF-R IgG 528. After several washings with salt solutions, EGF-R was displaced from the immobilized antibody by incubation with 0.1 mM mouse EGF dissolved in 20 mM HEPES, 1 mM EDTA, 130 mM NaCl. The final preparation contains homogenous EGF-R as demonstrated by silver stained SDS/PAGE and N-terminal sequencing analysis.

Cell Culture and Preparation of A431 Cell Membranes—Cell culture and preparation of A431 cell membranes were performed as described by Hubler *et al.* (*8*).

Tyrosine Kinase Assay—Standard reactions were performed in TGT-buffer [20 mM Tris/HCl pH 7.5, 10% (v/v) Glycerol, 0.05% (v/v) Triton X-100, 1 mM EDTA, 1 mM β-mercaptoethanol] containing 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (5×10^4 cpm/pmol) 0.1 mg/ml BSA, substrate protein in concentrations indicated and 10 fmol of purified, intact or proteinase-treated EGF-R at 37°C in a volume of 20 μl for 5 min. The polyions were added as indicated in the legends. The reactions were started by the addition of [γ-^{32}P]ATP and stopped by the addition of 20 μl of Laemmli buffer followed by boiling for 5 min (*9*). The samples were

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Abbreviations: EGF, epidermal growth factor; EGF-R, EGF receptor; IC_{50}, inhibitor concentration at which the half maximal inhibition was observed; FSBA, fluorosulfonylbenzoyladenosine; BSA, bovine serum albumin; TCA, trichloroacetic acid.

subjected to SDS/PAGE and autoradiography. The ^{32}P incorporation was determined by counting the Cerenkov radiation of phosphoproteins cut from dried gels. Kinetic parameter were determined by nonlinear regression analysis using ENZFITTER (BioSoft) and SIGMA PLOT (Jandel). The results presented are averaged from 3 independent assays.

Proteolytical Processing of A431 Cell Membranes and Generation of the 42 kDa Kinase—Aliquots of 10 μl of A431 cell membranes (0.2 mg protein) were incubated with proteinases at different concentrations, as indicated in the legend, for 30 min at 30°C in TGT-buffer. The proteolysis was terminated by the addition of 20 μl of Laemmli buffer followed by boiling for 5 min. The proteins were separated in polyacrylamide gel containing α-casein (1 mg/ml). The kinase activity was analyzed according to procedure of Kameshita and Fujisawa (*10*) with modifications. Briefly, after electrophoresis at 4°C, the gels were washed six times each for 15 min at 37°C in TGT-buffer, incubated for 30 min in 6 M urea and washed again six times. After incubation overnight at 37°C in 10 ml of TGT-buffer containing 1 μM [γ-^{32}P]ATP (10 μCi/ml) and 10 mM $MgCl_2$, the gels were extensively washed in 10% (w/v) TCA containing 0.5% (v/v) H_3PO_4, dried and subjected to autoradiography.

The immunoaffinity-purified EGF-R was proteolytically treated as follows: 100 fmol of enzyme was incubated with 2 fmol of trypsin or *S. griseus* proteinase in TGT-buffer for 10 min at 30°C in a total volume of 100 μl. To check the efficiency of the proteolytic degradation, a 5 μl aliquot of proteinase-treated EGF-R was autophosphorylated and subjected to SDS/PAGE as described above. The reaction was stopped by addition of 30 μl of proteinase inhibitor mixture containing 0.5 mg/ml BSA, 5 U/ml Aprotinin, 10 mM (4-amidinophenyl)methansulfonylfluoride (APMSF), 2 mM L-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK). To rule out effects caused by inactivation of the enzymes during incubation with proteinase, the holoenzyme was incubated in control assays under the same conditions in the presence of the proteinase inhibited prior to the incubation. The loss of activity of EGF-R incubated in the presence of the inhibited proteinase was subtracted from the obtained data.

Other Assays—The labeling of ATP-binding site of the EGF-R with [^{14}C]FSBA under non-reducing conditions was carried out as described previously (*11*). Protein concentration was measured according to the method of Lowry *et al.* (*12*).

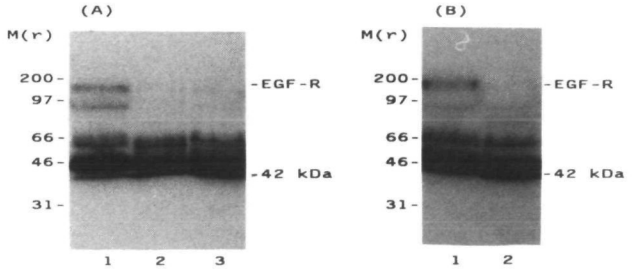

Fig. 1. **Detection of protein kinase activity in A431 cell membranes in renaturated gel.** Aliquots of 200 μg aliquots of freshly prepared A431 cell membranes were treated with 5 μg (A, lane 2) or 15 μg (A, lane 3) of trypsin, or with 2 μg of *Streptomyces griseus* proteinase (B, lane 2). In the lanes 1 (A and B), no proteinase treatment of A431 cell membranes was performed. The proteolysis was stopped by boiling with Laemmli buffer, and the samples were separated over a polyacrylamide gel containing α-casein as substrate. The gel was renaturated, incubated with [γ-^{32}P]ATP and subjected to autoradiography for 6 h at −80°C as described under "EXPERIMENTAL PROCEDURES."

RESULTS

Proteolytic Generation and Initial Characterization of 42 kDa Kinase—Proteinase treatment of crude A431 cell membranes resulted in depletion of the receptor and the concomitant generation of the kinetically active 42 kDa fragment. The kinetics of the depletion correlated closely with the loss of the ability of EGF to stimulate of phosphorylation of exogenous and endogenous substrates by the cell membrane preparation. The kinase could be visualized by "*in situ*" phosphorylation in renaturated polyacrylamide gel containing α-casein (Fig. 1). The proteolytically generated 42 kDa kinase was resistent to further degradation with trypsin even at higher concentrations of the proteinase

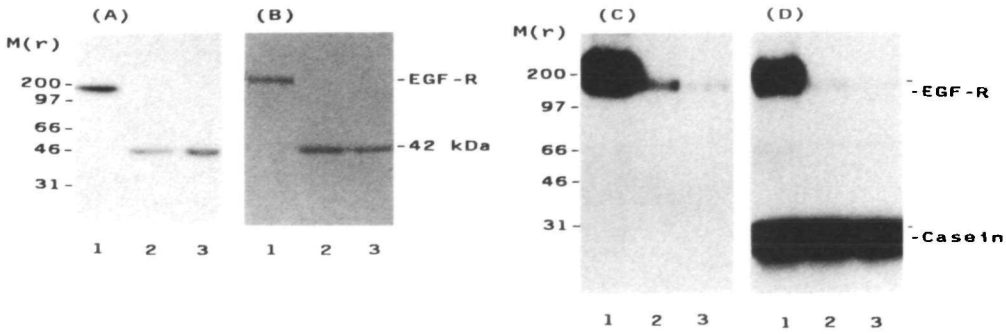

Fig. 2. **Analysis of "*in situ*" phosphorylation (A), ATP-binding site (B), autophosphorylation (C), and phosphorylation of α-casein (D) of EGF-R in the course of proteolytical processing with trypsin.** (A) Aliquots containing 1.25 pmol of immunoaffinity-purified EGF-R were incubated with trypsin (25 fmol) for 0, 10, and 30 min (lanes 1, 2, and 3, respectively). The samples were boiled with Laemmli buffer and submitted to electrophoresis in polyacrylamide gel containing α-casein. The renaturation of the gels, the "*in situ*" phosphorylation and autoradiography (for 48 h at −80°C) were performed as described above. (B) EGF-R (300 fmol) was incubated in the presence of 450 nCi of [^{14}C]FSBA for 60 min. Then trypsin (6 fmol) was added and incubation was continued. Aliquots corresponding to 100 fmol of EGF-R were removed after trypsin treatment for 0 min (lane 1), 10 min (lane 2), and 30 min (lane 3), mixed with Laemmli buffer and boiled. The samples were subjected to SDS/PAGE and the dried gel was exposed at −80°C for 14 days. (C and D) EGF-R (60 fmol) was incubated in the presence of trypsin (1.2 fmol). After trypsin treatment for 0 min (lane 1), 10 min (lane 2), and 30 min (lane 3), aliquots corresponding to 10 fmol of EGF-R were removed and investigated for the autophosphorylation (C) and phosphorylation of α-casein (D) at concentrations equal to K_m as described above. The probes were separated by SDS/PAGE, the gels were dried and exposed.

(Fig. 1A, lane 3). The treatment of A431 cell membranes with a non-specific *S. griseus* proteinase also resulted in depletion of EGF-R and generation of this fragment (Fig. 1B, lane 2).

Different preparations of EGF-R are known to vary in such properties as stimulation by EGF, response to polyions and affinity to substrate (*13*). Therefore, we used the immunoaffinity-purified receptor for further investigation and as a source of 42 kDa kinase. Tryptic processing of the homogenous preparation of EGF-R resulted in the generation of a 42 kDa fragment that exhibits kinase activity in the "*in situ*" phosphorylation assay (Fig. 2A) and maintains the ATP-binding site of the holoenzyme (Fig. 2B). The kinase was not capable of autophosphorylation but retained the full kinase activity of the EGF-R when assayed with α-casein as substrate (Fig. 2, C and D).

Substrate Specificity and Exogenous Kinase Activity of 42 kDa Kinase—Using proteins and synthetic polymers as substrates, we examined the phosphorylation mediated by EGF-R in the course of its tryptic processing. The effect of proteolysis on activity of EGF-R varied with different substrates. When the phosphorylation of poly (Glu^4, Tyr^1) was measured, a significant reduction of kinase activity in the course of tryptic degradation of EGF-R was seen. On the other hand, the phosphorylation of α-casein was not changed in the course of the proteolysis. Experiments performed with other substrates like histone H2B or poly (Glu^6, Ala^3, Tyr^1) revealed a reducing or increasing effect of proteolysis on the kinase activity of EGF-R (Fig. 3). K_m and V_{max} were calculated for the phosphorylation of the substrates by the EGF-R and the 42 kDa kinase. Comparison of the values obtained revealed that the observed alterations

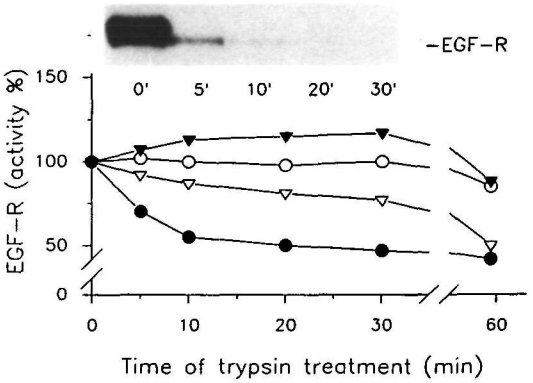

Fig. 3. **Alteration of EGF-R kinase activity to external substrates in course of tryptic generation of 42 kDa kinase.** EGF-R (100 fmol) was incubated with trypsin (2 fmol). At the times indicated, aliquots corresponding to 10 fmol of EGF-R were removed, the trypsin inhibited by addition of proteinase inhibitors and the kinase investigated for autophosphorylation and activity towards substrates. The concentrations of the substrates were equal to K_m values measured with the holoenzyme. The determination of phosphate incorporation and calculation of the kinase activity were performed as described under "EXPERIMENTAL PROCEDURES." The insert shows the autoradiogram demonstrating the autophosphorylation of the EGF-R at the indicated times. Symbols: open circles, α-casein; filled circles, poly(Glu^4, Tyr^1); open triangles, histone H2B; filled triangles, poly(Glu^6, Ala^3,Tyr^1).

TABLE I. **Kinetic parameters of phosphorylation of substrates by EGF-R and 42 kDa kinase.** The 42 kDa kinase was obtained by treatment of EGF-R with trypsin. Phosphorylation of substrates and determination of the kinase activity were performed as described in "EXPERIMENTAL PROCEDURES." The kinetic parameters were determined by nonlinear regression analysis and are reported as averages of 3 independent assays.

Substrate	42 kDa kinase		EGF-R	
	K_m (μM)	V_{max} (mol P_i/min)	K_m (μM)	V_{max} (mol P_i/min)
Poly(Glu^4, Tyr^1)	0.02	0.15	0.011	0.52
α-Casein	1.7	0.3	1.5	0.21
Poly(Glu^6, Ala^3, Tyr^1)	0.03	0.4	0.016	0.7
Histone H2B	7.2	0.012	4.8	0.03

TABLE II. **Inhibition studies of EGF-R and 42 kDa kinase.** The 42 kDa kinase was obtained by proteolytical processing of EGF-R with trypsin (*) or *Streptomyces griseus* proteinase (**). The phosphorylation of poly(Glu^4, Tyr^1) (at concentrations equal to respective K_m values), calculation of the kinase activity and determination of inhibitory constants were performed as described in "EXPERIMENTAL PROCEDURES" and are reported as averages of 3 independent assays.

Inhibitor	IC_{50} (μM)		
	42 kDa kinase		EGF-R
	Trypsin*	*S.g.* proteinase**	
Genistein	410	35	32
Methyl-2,5-dihydroxycinnamate	15	2.7	10
Tyrphostin	150	12	25
Piceatanol	20	35	25

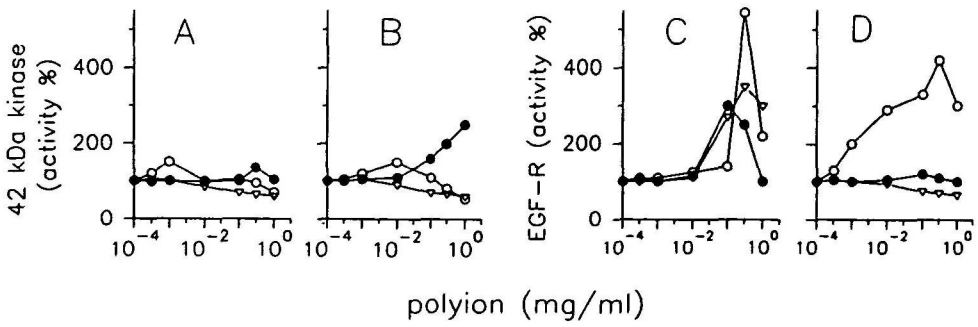

Fig. 4. **Comparison of effects of polyionic modulators on the activity of 42 kDa kinase and of EGF-R.** The 42 kDa kinase was obtained by treatment of EGF-R with trypsin. The concentrations of polyions were adjusted by their addition directly before the start of the reaction. The substrates were phosphorylated at concentrations equal to the respective K_m values, and the activity was determined as described under experimental procedures. The obtained values are presented as percentages of controls (no polyion treatment) and are the averages of 3 independent assays. The activity of 42 kDa kinase as a function of added polyions is shown in A and B and that of EGF-R in C and D. The diagrams demonstrate the phosphorylation of poly(Glu^4, Tyr^1) (A and C) and of α-casein (B and D). Symbols: open circles, poly(Lys); filled circles, protamine; open triangles, spermidine.

of the substrate specificity result mainly from changes of V_{max} and, to a lesser extent, of K_m (Table I).

The general pattern of the activation or reduction of the phosphorylation was changed when the proteolysis of EGF-R was performed with *S. griseus* proteinase. In this case the phosphorylation of α-casein and poly(Glu^4, Tyr^1) was reduced (approx. 20–30% and 60–70%, respectively) compared with the holoenzyme.

Regulation of 42 kDa Kinase Activity by Polyionic Compounds—The kinase activity of EGF-R was shown to be very potently modulated by polyionic compounds (*14*). After the limited proteolysis, a reduction, amplification or inversion of polyion effects was observed. The cationic protamine sulfate and poly(Lys) enhanced significantly the phosphorylation of poly(Glu^4, Tyr^1) by EGF-R with maximal effect at 0.1–0.3 mg/ml. Tryptic digestion of the EGF-R reduced the stimulating effects of both compounds, and in the case of poly(Lys) the stimulation was seen at a concentration of 10 μg/ml. The polycationic spermidine, which activates phosphorylation of poly(Glu^4,Tyr^1) by EGF-R (at a concentration of 0.5–1.5 mg/ml), did not stimulate the phosphorylation by 42 kDa kinase, but slightly inhibited it (Fig. 4, A and C). The negatively charged dextran sulfate, which is a strong inhibitor of phosphorylation of poly(Glu^4, Tyr^1) by EGF-R, showed the same inhibiting effect on the 42 kDa kinase (IC_{50} values for both kinases were 3.2 and 3.7 ng/ml, respectively).

Numerous reports demonstrate that the polyion-mediated modulation of kinase activity is specific for each combination of enzyme and substrate (*15*). However, similarly to the observations with poly(Glu^4, Tyr^1), strong activation of EGF-R by poly(Lys) in a broad concentration range was also observed with α-casein as substrate. Poly(Lys) activation of 42 kDa kinase was rather modest (1.5-fold *versus* 4-fold for EGF-R) and the optimal activating concentration was shifted to 3–10 μg/ml. At concentrations of 0.1–1.5 mg/ml poly(Lys) inhibited strongly the phosphorylation of α-casein by 42 kDa kinase. On the other hand protamine, which has no appreciable effect on the phosphorylation of α-casein by EGF-R, enhanced significantly the phosphorylation by 42 kDa kinase up to 2–2.5-fold (compared with no polyion treated kinase). Spermidine showed a weak inhibiting effect on α-casein phosphorylation by both EGF-R as well as by 42 kDa kinase in a wide concentration range of 0.15–5 mg/ml (Fig. 4, B and D).

Dextran sulfate enhanced phosphorylation of α-casein by EGF-R (3–3.5-fold activation at 1 μg/ml) but higher concentrations were strongly inhibiting ($IC_{50} = 0.1$ mg/ml). On the other hand, no stimulating effect on 42 kDa kinase could be observed, while the IC_{50} was not altered.

Action of Tyrosine Kinase Inhibitors—The tryptic digestion altered the susceptibility of EGF-R to inhibition by tyrosine kinase inhibitors. The changed response appeared both with compounds competing with substrate and with compounds competing with ATP. Genistein, an ATP competitor (*16*), inhibited phosphorylation of poly(Glu^4, Tyr^1) by EGF-R with an IC_{50} of 32 μM, and tryptic digestion shifted the IC_{50} to 410 μM. Tyrphostin, a substrate competitor (*17*), similarly showed an inhibiting effect at higher concentrations after digestion of the enzyme. On the other hand, inhibition by methyl-2,5-dihydroxycinnamate, which competes for the substrate-binding site (*18*), and by piceatannol, which competes for the ATP-binding site (*19*), was only marginally altered after tryptic digestion of EGF-R. It is noteworthy that 42 kDa kinase obtained by proteolysis with *S. griseus* proteinase revealed other responsivity to the investigated inhibitors. The inhibition studies are summarized in Table II.

DISCUSSION

A proteinase-resistant domain of EGF-R, maintaining its tyrosine kinase activity and ATP-binding site, was produced by limited proteolysis *in vitro*. The obtained fragment appears to be linked to other domains of EGF-R by regions which are relatively sensitive to proteolysis and are common points of attack for a variety of proteinases. Indeed, in the course of limited proteolysis of EGF-R, three tryptic fragments could be obtained: (1) a 120 kDa domain which contains EGF-binding and protein kinase C phosphorylation (Thr 654) sites (*20, 21*); (2) an adjacent 40 kDa fragment which contains a lysine residue (Lys 721) covalently labeled with FSBA and a sequence, GlyXGlyXX-Gly (residues 695–700), involved in ATP-binding (*22, 23*); and (3) a terminal 15 kDa portion of the EGF-R containing the autophosphorylation sites (*6*). Thus, one of the trypsin cleavage sites resides in the linear stretch of 41 residues between Thr 654 and Gly 695. The other proteinase-susceptible region of EGF-R, connecting the kinase domain and autophosphorylation sites, contains cleavage sites for chymotrypsin, elastase, calpain, and trypsin (*1*). Proteolysis experiments with calpain [cleavage site at Lys 1037 (*24*)] defines this region more precisely (Fig. 5). Many tyrosine kinases share a similar structural organization with a similarly sized (40–43 kDa) catalytic domain which is relatively resistent to limited proteolysis (*25, 26*). Thus, it could be not ruled out that the 42 kDa kinase detected in

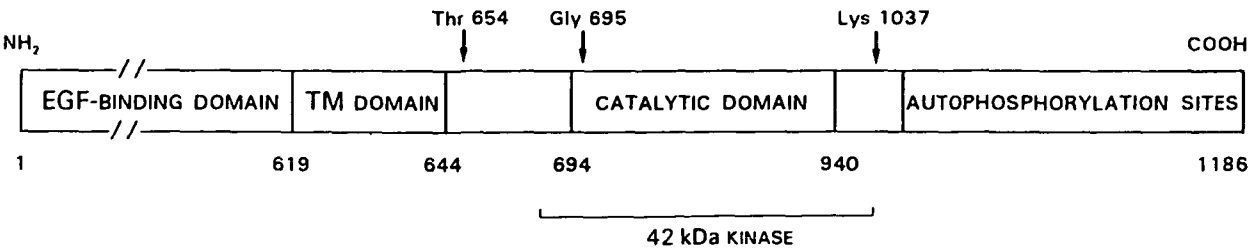

Fig. 5. **Schematic diagram showing the structural features of EGF-R and the start and end of 42 kDa kinase.** The receptor is depicted as a bar, with amino acid numbers taken from Ref. *30*. Indicated are: Thr 654, threonine residue phosphorylated by protein kinase C; Gly 695, start of recognizable homology with catalytic domain of tyrosine kinases; Lys 1037, calpain cleavage site; TM = transmembrane.

renaturated gel (Fig. 1) results from cleavage of other kinase(s) too, more particularly as it was seen also in membrane preparation not treated with proteinases (Fig. 1, A and B, lanes 1). On the other hand, proteinase treatment of homogenous EGF-R under the same reaction conditions generated 42 kDa kinase with the same kinetics. This finding led us to conclude that the increase of 42 kDa kinase activity seen in course of proteolytical processing of membranes results at least partly from degragdation of EGF-R.

As shown in this study, the 42 kDa kinase obtained from homogenous preparation of EGF-R was not capable of autophosphorylation. The contradiction to the data of Basu *et al.* seems to be derived from differences in purity and perhaps the conditions of the assay systems (*6*). When the 42 kDa kinase was compared with the holoenzyme, differences between their biochemical properties were observed. The kinase is capable of phosphorylation of a number of proteins and synthetic polymers previously reported to be substrates for EGF-R. Like the holoenzyme, it prefers rather acidic substrates. The differences in the substrate affinity could be elucidated on the basis of the active site structure of prototype tyrosine kinase: insulin receptor (*27*). The catalytical domain loops of insulin receptor contain conserved tyrosine kinase-specific arginine residues, Arg 1089 (also arginine in EGF-R) and Arg 1092 (corresponding to lysine in EGF-R), which are potential contacts for negatively charged residues of preferred substrates (absent in histone H2B). On the other hand, Arg 1131, which is also conserved, and Arg 1155 (lysine in EGF-R) stabilize the non-inhibiting conformation of the activation loop by interaction with autophosphorylated tyrosine residues. The acidic residues of the substrates [α-casein, poly(Glu^4, Tyr^1) and poly(Glu^6, Ala^3, Tyr^1)] could mimick the effect of the phosphate and further stabilize the non-inhibiting conformation. Nevertheless, substrate-dependent changes in the kinetic parameters of both kinases were observed. These changes presumably reflect differences in the accessibility of the high-order protein determinants or in the proximity of their catalytic domains. The fact that the response of the 42 kDa kinase to polycations is strongly reduced compared to EGF-R demonstrates that the activation mediated by substrate modulators (chaperones) does not occur exclusively at the substrate level as suggested previously (*15*).

Our observations indicate that the domains (structures) of EGF-R localized in the C- or N-terminus, which are not present in the 42 kDa kinase, determine the substrate specificity and mediate the interactions with the chaperones. This idea is corroborated by the following findings. First, Khazaie *et al.* have shown that C-terminal deletions of EGF-R led to alterations of its substrate specificity and changes of its transforming capacity (*28*). Second, in the regulation of the EGF-R and 56 kDa kinase domain containing residues 644-1134 of the receptor, both kinases showed a differential response on poly(Lys) and heparin respectively to autophosphorylation and phosphorylation of substrate (*13*).

The inhibition studies also agree with the results presented here. For the recognition of relatively small molecules like kinase inhibitors, the holoenzyme requires the participation of domains located distal to its catalytic site. The lack of these domains in the 42 kDa kinase contributes to the comparatively higher IC_{50} value found for genistein and tyrphostin. A similar type of interaction was found previously between the C-subunit of the cAMP-dependent kinase and small peptide inhibitors or peptide substrates. The recognition of the small molecules involves large, surface-localized domains of the enzyme (*29*).

On the basis of our observations and published data, it appears that the proteolysis of EGF-R does not lead to a simple prolongation of its activation. The kinase generated in the course of the proteolytical degradation differs from the holoenzyme.

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