Inhibition of Tyrosine Protein Kinases by Halomethyl Ketones†

J. Navarro, M. Abdel Ghany, and E. Racker*

ABSTRACT: A chloromethyl ketone derivative of lactic acid was shown to inhibit protein phosphorylation in plasma membranes of Ehrlich ascites tumor cells [Johnson, H. J., Zimniak, A., & Racker, E. (1982) *Biochemistry 21*, 2984–2989]. We now show that this inhibitor as well as three halomethyl ketone derivatives of amino acids and peptides specifically inhibits tyrosine protein kinase activity in intact plasma membranes and Triton extracts of plasma membrane of A-431 tumor cells. The most effective inhibitor is a bromomethyl ketone derivative of leucine that inhibits the phosphorylation of a protein that migrates to the same position as the EGF receptor in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Inhibition of phosphorylation took place in the presence or absence of added EGF, and the inhibitor did not interfere with the binding of EGF to the receptor nor with the dephosphorylation of the EGF-stimulated phosphoprotein. EGF-dependent phosphorylation in a Triton extract of plasma membranes from normal placenta was considerably less sensitive to the bromomethyl ketone derivative of leucine. The tyrosine protein kinase activity of the transformation gene product of Fujinami virus was particularly sensitive to the bromomethyl ketone derivative of leucine, while the *src* gene product of Rous sarcoma virus was comparatively less sensitive. The bromomethyl ketone inhibitor interfered with the phosphorylation of the EGF receptor by [γ-^{32}P]-8-azido-ATP but much less with the light-sensitive binding. This observation and the lack of interference with EGF binding suggest that the inhibitor interacts with the protein kinase portion of the receptor complex.

An enhanced tyrosine phosphorylation of proteins in the plasma membrane of A-431 human epidermoid carcinoma cells appears to be an early event triggered by the interaction of EGF[1] with its receptor (Ushiro & Cohen, 1980). Transformation of cell lines by various tumor viruses also induces an increase of tyrosine phosphorylation of specific virus gene products (Eckhart et al., 1979; Witte et al., 1980; Erikson et al., 1980; Bishop, 1981; Ross et al., 1981). It thus appears that in these instances an increased tyrosine phosphorylation is associated with changes in the pattern of cell growth. Purified EGF receptor and at least some of the purified viral gene products were shown to contain the protein kinase activity that gives rise to tyrosine phosphorylation, and several proteins have been isolated that serve as substrates for these enzymes (Chinkers & Cohen, 1981; Kudlow et al., 1981). However, only a little is known about how these events relate to the changes in physiology of cells that are exposed to the growth factor or to the transforming virus.

Among the changes induced by both EGF (Diamond et al., 1978) and transformation (Racker, 1976) is an increased rate of glycolysis. It was shown recently (Johnson et al., 1982) that a chloromethyl ketone derivative of lactic acid (iLac-CH_2Cl) inhibits glyclysis and hexokinase activity, as well as the phosphorylation of proteins in the plasma membrane of Ehrlich ascites tumor cells. In view of a possible role of tyrosine phosphorylation in a variety of cell functions, a more extensive study of the effect of various halomethyl ketone derivatives was undertaken.

Experimental Procedures

Materials. Acetylalanylalanylphenylalanylthreonine chloromethyl ketone, acetylglycylglycylalanylphenylalanine chloromethyl ketone, benzyloxycarbonylphenylalanine chloromethyl ketone, and benzyloxycarbonyltryptophan chloromethyl ketone were gifts from Dr. J. C. Powers (Georgia Institute of Technology, Atlanta, GA). Acetylphenylalanylglycylalanylleucine chloromethyl ketone, benzyloxycarbonylalaninyl chloromethyl ketone, leucine chloromethyl ketone, benzyloxycarbonylleucine diazomethyl ketone, and *tert*-butyloxycarbonylleucine bromomethyl ketone were provided by Dr. C. Kettner from Du Pont, Wilmington, DE. [γ-^{32}P]ATP and Na^{125}I were purchased from ICN, Irvine, CA. Histone II, Triton X-100, Hepes, γ-globulins (Cohn fraction II), ouabain, BSA, phosphoserine, and phosphothreonine were obtained from Sigma Chemical Co., St. Louis, MO. Phosphotyrosine was prepared by M. Spector. PEG 6000 was purchased from Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH; [γ-^{32}P]-8-azido-ATP was generously supplied by Dr. B. Haley, University of Wyoming, Laramie, WY.

Growth of A-431 Cells and Preparation of Membrane Fraction. A-431 human epidermoid carcinoma cells (provided by Dr. G. Todaro) were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Plasma membranes were isolated by the method described by Thom et al. (1977) and kept at −70 °C. Solubilization of plasma membranes was performed as described by Cohen et al. (1980). Briefly, 7.5 mg/mL of protein was solubilized with 1% Triton X-100, 10% glycerol, and 20 mM Hepes (pH 7.4). This mixture was incubated for 20 min at room temperature and then centri-

† From the Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. *Received April 28, 1982.* This investigation was supported by Grant BC-156 from the American Cancer Society and Grants CA-14454 and CA-08964, awarded by the National Cancer Institute, DHHS.

[1] Abbreviations: Ac-Ala-Ala-Phe-Thr-CH_2Cl, acetylalanylalanylphenylalanylthreonine chloromethyl ketone; Ac-Gly-Gly-Ala-Phe-CH_2Cl, acetylglycylglycylalanylphenylalanine chloromethyl ketone; Z-Phe-CH_2Cl, benzyloxycarbonylphenylalanine chloromethyl ketone; Z-Trp-CH_2Cl, benzyloxycarbonyltryptophan chloromethyl ketone; Ac-Phe-Gly-Ala-Leu-CH_2Cl, acetylphenylalanylglycylalanylleucine chloromethyl ketone; Leu-CH_2Cl, leucine chloromethyl ketone; Z-Leu-CHN_2, benzyloxycarbonylleucine diazomethyl ketone; Boc-Leu-CH_2Br, *tert*-butyloxycarbonylleucine bromomethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperizine-*N*′-2-ethanesulfonic acid; 8-azido-ATP, 8-azidoadenosine triphosphate; BSA, bovine serum albumin; PEG-6000, poly(ethylene glycol) 6000; EGF, epidermal growth factor; iLac-CH_2Cl, 2-*O*-(isobutoxycarbonyl)lactate chloromethyl ketone; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Effect of Halomethyl Ketone Derivatives of Amino Acids and Peptides on Protein Phosphorylation in Triton Extracts of Plasma Membranes of A-431 Cells[a]

additions	−EGF (pmol of ^{32}P incorporated × min^{-1} × mg of $protein^{-1}$)	% inhibition	+EGF (pmol of ^{32}P incorporated × min^{-1} × mg of $protein^{-1}$)	% inhibition
none	7.3		20.2	
Ac-Ala-Ala-Phe-Thr-CH_2Cl	6.6		19.5	
Ac-Gly-Gly-Ala-Phe-CH_2Cl	7.7		18.3	
Ac-Phe-Gly-Ala-Leu-CH_2Cl	5.2	29	8.3	59
Z-Ala-CH_2Cl	4.1	44	5.9	71
Leu-CH_2Cl	8.1		16.9	
Z-Leu-CHN_2	6.0		17.0	
Boc-Leu-CH_2Br	2.3	69	7.0	65
Z-Phe-CH_2Cl	6.2		16.0	
Z-Trp-CH_2Cl	6.6		19.0	

[a] The compounds (dissolved in ethanol) were added to the reaction mixture in a final volume of 70 μL, containing 20 mM Hepes (pH 7.4), 0.01% BSA, 1 mM $MnCl_2$, and Triton extracts of A-431 plasma membranes (63 μg of protein). The final concentration of these compounds was 400 nmol/mg of protein. After 10-min incubation at room temperature, 100 ng of EGF was added and the mixture was incubated for another 10 min. The tubes were then kept at 0 °C for 10 min and the reaction was always started by addition of [γ-^{32}P]ATP (15 μM, 4 μCi) and terminated as described under Experimental Procedures.

fuged at 100000*g* for 60 min.

Phosphorylation Assay. Phosphorylation of proteins was performed as described by Carpenter et al. (1979). The incubation system contained (in a final volume of 70 μL) 20 mM Hepes buffer (pH 7.4), 0.01% BSA, plasma membrane, or Triton extract (15–150 μg of protein), 100 ng of EGF, 1 mM $MnCl_2$, 1 mM ouabain (only in those samples containing intact plasma membranes), and [γ-^{32}P]ATP (10–20 μM, 2–4 μCi). Halomethyl ketone derivatives were added in alcoholic solutions; an equivalent volume of ethanol was added to a control sample. The reaction mixture was incubated at room temperature for 10 min with and without halomethyl ketone derivatives and with or without EGF. The mixture was then kept at 0 °C for 10 min before the reaction was initiated by addition of [γ-^{32}P]ATP (15 μM, 2 μCi, or as otherwise indicated). The reaction was usually terminated after 3 min at 0 °C by applying an aliquot of the reaction mixture to a Whatman 3MM filter paper (2 × 2 cm) that was then placed into ice-cold 10% trichloroacetic acid containing 10 mM pyrophosphate and gently shaken for 1 h with three changes of the washing fluid. The filter papers were dried and the radioactivity was measured in a liquid scintillation counter.

Gel Electrophoresis and Autoradiography. The membrane proteins were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5% acrylamide) by the method of Laemli (1970). The slab gels were dried and autoradiography was performed according to the method described by Cohen et al. (1980).

Hydrolysis and Electrophoresis of ^{32}P-Labeled Membrane. To the ^{32}P-labeled membranes 10% trichloroacetic acid–10 mM sodium pyrophosphate and 100 μg of BSA were added. After incubation for 1 h at 0 °C the mixture was centrifuged at 3000 rpm and the pellet was washed 3 times with 10% trichloroacetic acid–10 mM sodium pyrophosphate. The final pellet was washed with ethanol and dried under vacuum. The material was dissolved in 40 μL of 0.1 N NaOH and transferred to glass test tubes containing 0.5 mL of 6 N HCl. The tubes were sealed under vacuum and the material was hydrolyzed at 100 °C for 1 h. The hydrolysates were lyophilized, resuspended in 30 μL of water and subjected to electrophoresis on Whatman 3MM paper for 3 h at 3000 V at pH 3.5 (5% acetic acid–0.5% pyridine). Phosphotyrosine, phosphothreonine, and phosphoserine (10 μg each) were used as markers. The amino acids were visualized with ninhydrin. The radioactivity on the paper was measured by excising the spots, bleaching with 6% H_2O_2 (Casnellie et al., 1982), and counting in a liquid scintillation counter.

Isolation and Iodination of EGF. Epidermal growth factor was prepared by the method of Savage & Cohen (1972). Iodination of EGF was performed according to the Chloramine T method of Cuatrecasas & Hollenberg (1976). The final specific activity of [^{125}I]EGF was 60 000 cpm/ng.

Binding of [^{125}I]EGF to Triton Extracts of Membranes. Assays for soluble [^{125}I]EGF–receptor complexes from A-431 cells were performed as described by Cohen et al. (1980). The reaction mixture (200 μL) contained 20 mM Hepes buffer (pH 7.4), 0.1% BSA, 1.67×10^{-8} M [^{125}I]EGF, and a Triton extract of membranes (150 μg of protein). The assay mixture was incubated for 30 min at room temperature and terminated by addition of 0.5 mL of 0.1% γ-globulin in 0.1 M sodium phosphate buffer (pH 7.4) and then 0.5 mL of 20.4% poly(ethylene glycol) 6000. This mixture was vortexed and filtered through EHWP Millipore filters. Nonspecific binding was measured in the presence of a 250-fold molar excess of unlabeled EGF. Nonspecific binding was about 20%.

Results and Discussion

Effect of Halomethyl Ketone Derivatives on Protein Phosphorylation in Plasma Membranes of A-431 Cells. Protein phosphorylation in the presence and absence of EGF by intact plasma membrane and Triton extracts of membranes of A-431 cells as a function of time and the amount of protein was qualitatively the same as those previously reported by Carpenter et al. (1979) and Cohen et al. (1980). The stimulation mediated by EGF in Triton extracts of membranes from A-431 cells resulted in an increase in V_{max} rather than in a change in the apparent K_m for ATP (data not shown). For most inhibitor studies the protein concentration was usually kept below 100 μg/70 μL, and the reaction was stopped after 3 min.

Since previous work in this laboratory has shown iLac-CH_2Cl inhibits protein phosphorylation in plasma membranes from Ehrlich ascites cells (Johnson et al., 1982), we have examined the effect of iLac-CH_2Cl on EGF-stimulated kinase activity of plasma membrane from A-431 cells. Figure 1 shows that the inhibition of protein phosphorylation in both control and EGF-stimulated kinase activity depended on the concentration of iLac-CH_2Cl. This observation led us to carry out a more extensive study with other halomethyl ketone derivatives. The effect of various amino acids and peptide de-

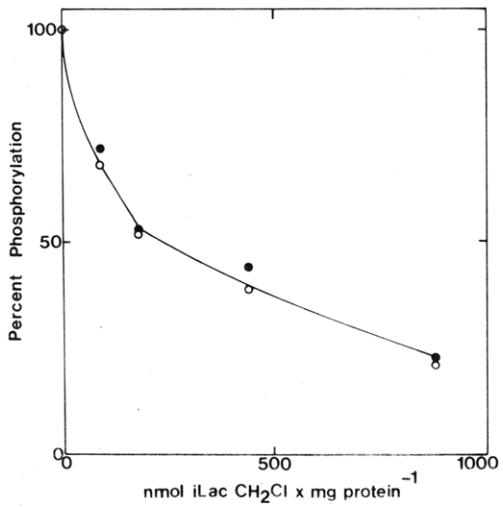

FIGURE 1: Inhibition of protein phosphorylation by iLac-CH_2Cl in plasma membranes from A-431 cells. A Triton extract of membranes was incubated with [γ-^{32}P]ATP (13 μM, 2 μCi) at 0 °C for 15 min. The concentration of protein per reaction tube was 50 μg. The reaction mixture was terminated as described under Experimental Procedures. In control (○) and EGF-stimulated phosphorylation (●) 100% activity was 14.5 and 32.6 pmol of ^{32}P incorporated × 15 min^{-1} × mg of protein^{-1}, respectively.

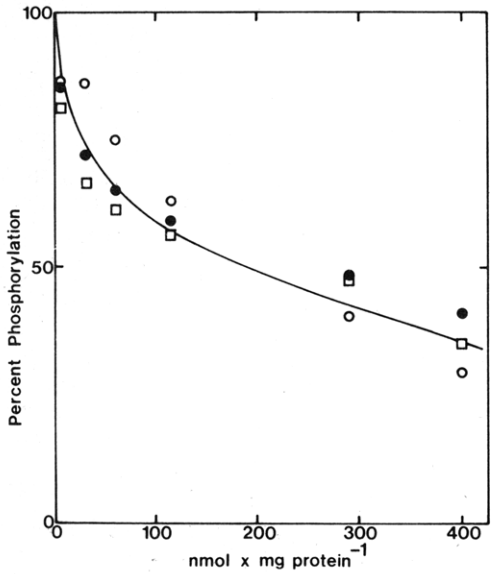

FIGURE 2: Inhibition of EGF-dependent phosphorylation by Ac-Phe-Gly-Ala-Leu-CH_2Cl, Z-Ala-CH_2Cl, and Boc-Leu-CH_2Br. The reaction was as described in Table I, except that the concentration of inhibitors was varied as indicated. The value for 100% phosphorylation was 20.2 pmol of ^{32}P incorporated × 15 min^{-1} × mg of protein^{-1}. Assays carried out (○) in the presence of Ac-Phe-Gly-Ala-Leu-CH_2Cl, (●) in the presence of Z-Ala-CH_2Cl, and (□) in the presence of Boc-Leu-CH_2Br.

rivatives on protein phosphorylation is shown in Table I. The concentrations are expressed as nanomoles per milligram of protein, as is customary for hydrophobic inhibitors. Of the compounds listed, three significantly inhibited phosphorylation. The most potent inhibitor was the bromomethyl ketone derivative of leucine, which inhibited about equally the phosphorylation in the absence and presence of EGF. The two other compounds, chloromethyl ketone derivatives of alanine and of phenylalanylglycylalanylleucine, inhibited the EGF-stimulated phosphorylation preferentially. It is important to stress that several other chloromethyl ketone derivatives listed in Table I had little or no effect, thus ruling out a nonspecific interaction of the halomethyl ketone group with, for example,

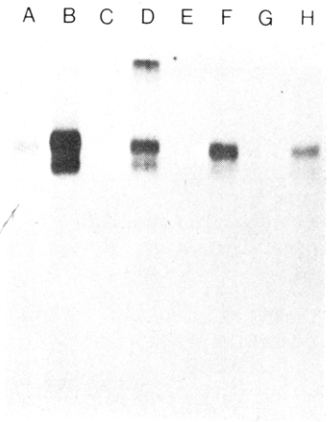

FIGURE 3: Effect of inhibitors on the phosphorylation of plasma membrane proteins of A-431 cells. A Triton extract of membranes was incubated with [γ-^{32}P]ATP (15 μM, 3 μCi) at 0 °C for 3 min. Samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5% acrylamide), Coomassie blue staining, and autoradiography as described under Experimental Procedures. Lanes A, C, E, and G were samples incubated without EGF and lanes B, D, F, and H with EGF. Lanes C and D were incubated in the presence of Boc-Leu-CH_2Br, lanes E and F in the presence of Z-Ala-CH_2Cl, and lanes G and H in the presence of Ac-Phe-Gly-Ala-Leu-CH_2Cl. The concentration of the inhibitors was 0.4 μmol/mg of protein.

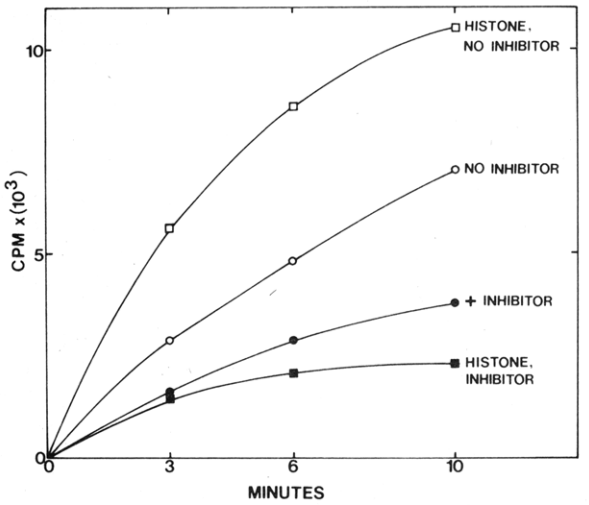

FIGURE 4: Inhibition of the EGF-stimulated phosphorylation by Boc-Leu-CH_2Br in intact plasma membrane from A-431 cells. Intact plasma membranes (40 μg of protein) were incubated with [γ-^{32}P]ATP (15 μM, 3 μCi) in the presence and absence of histone II (1 mg/mL). Aliquots of 20 μL were taken at the indicated times and the amount of phosphorylated protein was determined as described under Experimental Procedures. The concentration of Boc-Leu-CH_2Br was 600 nmol/mg of protein.

an SH group of the receptor protein kinase. The inhibition of EGF-stimulated phosphorylation by the three active compounds was quite similar and dependent on concentration, as shown in Figure 2. $NaDodSO_4$–polyacrylamide gel electrophoresis showed that EGF increased the phosphorylation of bands at 170000 and at 150000 daltons (Figure 1). According to Cohen et al. (1982) the doublet represent two different forms of the EGF receptor. The three inhibitor compounds markedly inhibited the phosphorylation of the 170000- and 150000-dalton bands (Figure 3) in both the control and EGF-stimulated phosphorylation. Furthermore, Figure 4 shows that Boc-Leu-CH_2Cl also inhibits phosphorylation of proteins in the intact plasma membranes of A-431 cells. Since Carpenter et al. (1979) have shown that histone serves as a substrate for the EGF-stimulated kinase, we have examined the effect of Boc-Leu-CH_2Cl on the phosphorylation of histone.

Table II: Effect of Kinase Inhibitors on the Binding of [^{125}I]EGF[a]

additions	specific binding (pmol/mg of protein)	% inhibition
none	1.49	
Boc-Leu-CH_2Br	1.42	4.6
Z-Leu-CHN_2	1.48	0.5
Leu-CH_2Cl	1.46	1.5
Ac-Phe-Gly-Ala-Leu-CH_2Cl	1.38	6.7
Z-Ala-CH_2Cl	1.35	8.8

[a] The incubation mixture contained 20 mM Hepes (pH 7.4), 0.1% BSA, 1.67×10^{-8} M [^{125}I]EGF, and Triton extract of A-431 plasma membranes (160 μg of protein) in a final volume of 200 μL. The concentration of inhibitors was 460 nmol/mg of protein. This reaction mixture was incubated for 30 min at room temperature. The reaction was terminated and analyzed as described under Experimental Procedures. Corrections were made for nonspecific binding, which was about 20%.

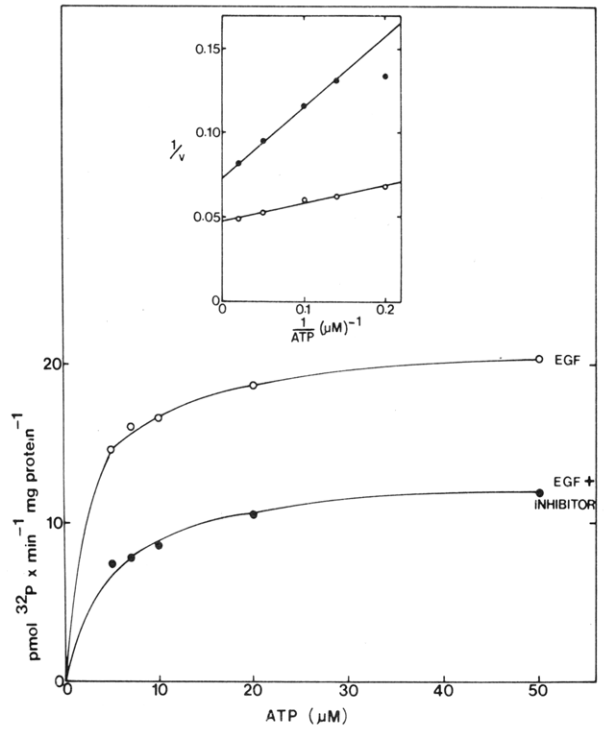

FIGURE 5: Effect of Boc-Leu-CH_2Br on EGF-dependent phosphorylation as a function of ATP concentration. The assay was carried out at 0 °C for 4 min as described under Experimental Procedures. The insert shows a Lineweaver–Burk plot of the data. The concentration of Boc-Leu-CH_2Br was 170 nmol/mg of protein.

The data in Figure 4 show that Boc-Leu-CH_2Cl strongly inhibits the phosphorylation of histone by intact plasma membranes.

Since Z-Ala-CH_2Cl and Ac-Phe-Gly-Ala-Leu-CH_2Cl appear to be inhibiting EGF-stimulated phosphorylation preferentially (see Table I), the effect of these compounds on EGF binding to the membrane was explored. The extent of binding was tested in the presence of 1.67×10^{-8} M [^{125}I]EGF, which corresponds to half-maximal binding of EGF to the solubilized plasma membrane from A-431 cells (Carpenter, 1979). Under these conditions the extent of binding should be very sensitive to an inhibitor. As shown in Table II none of the derivatives tested significantly affected the binding of EGF to its receptor. In addition, Figure 5 shows that the effect of Boc-Leu-CH_2Br (170 nmol/mg of protein) was on V_{max} and also on the apparent K_m for ATP.

Table III: Protection by EGF against Inhibition of Membrane Phosphorylation by Halomethyl Ketone Derivatives[a]

additions		^{32}P incorporation (cpm)	% inhibition
1st incubation	2nd incubation		
none		3703	
EGF		8134	
Boc-Leu-CH_2Br	EGF	3304	59
EGF	Boc-Leu-CH_2Br	4620	43
Z-Ala-CH_2Cl	EGF	3458	57
EGF	Z-Ala-CH_2Cl	6307	22
Ac-Phe-Gly-Ala-Leu-CH_2Cl	EGF	4263	47
EGF	Ac-Phe-Gly-Ala-Leu-CH_2Cl	5243	35

[a] A Triton extract of A-431 plasma membranes (72 μg of protein) was incubated with inhibitor or EGF for 10 min at room temperature, followed by a second incubation for 10 min with EGF or inhibitor. The reaction was initiated by addition of [γ-^{32}P]ATP (10 μM, 3 μCi) and incubated for 3 min at 0 °C. The concentration of inhibitor was 350 nmol/mg of protein.

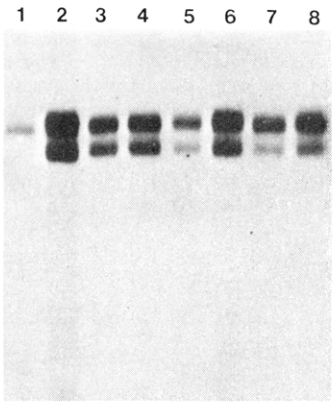

FIGURE 6: Protection by EGF against inhibition of the phosphorylation of EGF receptor by halomethyl ketone derivatives. Triton extracts of A-431 membranes (72 μg of protein) were incubated with inhibitor or EGF for 10 min at room temperature, followed by a second incubation for 10 min with EGF or inhibitor. The reaction was initiated by addition of [γ-^{32}P]ATP (10 μM, 3 μCi) and incubated for 3 min at 0 °C. The samples were then subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, Coomassie blue staining, and autoradiography as described under Experimental Procedures. Lane 1 is a sample in the absence of EGF and inhibitor, lane 2 is a sample in the presence of EGF, lane 3 is a sample treated with Boc-Leu-CH_2Cl followed by EGF, lane 4 is a sample treated with EGF followed by Boc-Leu-CH_2Cl, lane 5 is a sample treated with Z-Ala-CH_2Cl followed by EGF, lane 6 is a sample treated with EGF followed by Z-Ala-CH_2Cl, lane 7 is a sample treated with Ac-Phe-Gly-Ala-Thr-CH_2Cl followed by EGF, and lane 8 is a sample treated with EGF followed by Ac-Phe-Gly-Ala-Thr-CH_2Cl. The concentration of inhibitors was 350 nmol/mg of protein.

Since the extent of phosphorylation in a crude extract is a function of both proteins kinase and protein phosphatase activities, the effect of the inhibitors on the latter was also examined as described by Carpenter et al. (1979). A large excess of Boc-Leu-CH_2Br (1 μmol/mg of protein) had no effect on the rate of dephosphorylation (data not shown).

Interaction of Halomethyl Ketones with EGF Receptor in the Presence and Absence of EGF and [γ-^{32}*P*]*-8-Azido-ATP.* As can be seen from Table III and Figure 6 EGF significantly protected against the inhibition by all three halomethyl ketone derivatives, particularly the alanylchloromethyl ketone. Since we have seen (Table II) that the binding of [^{125}I]EGF was not affected by the inhibitors, it seems likely that the protective effect of EGF is due to a conformational change of the receptor protein.

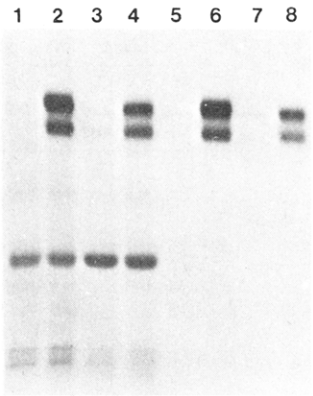

FIGURE 7: Effect of Boc-Leu-CH_2Br on the labeling of the EGF receptor by a photoaffinity derivative of ATP ([γ-^{32}P]-8-azido-ATP). Triton extracts of A-431 membranes (65 μg of protein) were incubated with and without EGF (100 ng) in the presence and absence of Boc-Leu-CH_2Cl, 20 mM Hepes pH 7.4, 1 mM MnCl, and 0.01% BSA. The reaction was initiated by addition of [γ-^{32}P]-8-azido-ATP (42 μM, 3.7 μCi) and incubated for 1 min at 0 °C in the presence and absence of ultraviolet light. The reaction was terminated by addition of sample buffer and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, Coomassie blue staining, and autoradiography as described under Experimental Procedures. 1–4 were samples exposed to ultraviolet light. 5–8 were samples in the absence of light. 1 and 5 were samples in the absence of both EGF and inhibitor. 2 and 6 were samples in the presence of EGF. 3 and 7 were samples in the presence of Boc-Leu-CH_2Br and the absence of EGF. 4 and 8 were samples in the presence of both EGF and Boc-Leu-CH_2Br. The final concentration of Boc-Leu-CH_2Br was 770 nmol/mg of protein.

Since the interaction between EGF and receptor activates protein kinase activity and since Boc-Leu-CH_2Br appears to affect V_{max} and K_m, its interaction with the active site of the protein kinase was examined. The Triton-extracted plasma membranes were exposed to [γ-^{32}P]-8-azido-ATP in the presence and absence of ultraviolet light with and without inhibitor. Figure 7 shows that [γ-^{32}P]-8-azido-ATP transferred radioactivity to the receptor protein both in the presence and in the absence of light. This finding indicates that [γ-^{32}P]-8-azido-ATP serves as a phosphate donor for the EGF receptor. Several others proteins of lower molecular weight were labeled in the light but not in the dark. Boc-Leu-CH_2Br appears to inhibit the labeling of the protein kinase by [γ-^{32}P]-8-azido-ATP in the absence and presence of light, but the extent of inhibition was less in the presence of light, which suggests that [γ-^{32}P]-8-azido-ATP still binds to the protein kinase in the presence of Boc-Leu-CH_2Br. The effect of the inhibitor therefore appears to be more pronounced on the phosphotransfer reaction than on the binding of azido-ATP. These data support the view that the protein kinase activity resides in the receptor protein complex. After submission of this manuscript a report appeared by Buhrow et al. (1982) showing that the 170K EGF receptor can be affinity labeled with 5′-(*p*-sulfonylbenzoyl)adenosine, which is in agreement with our findings.

Specificity of Inhibition by Boc-Leu-CH_2Br of Tyrosine Phosphorylation. It can be seen from Figure 8 and Table IV that Triton extracts of plasma membranes of A-431 cells were phosphorylated at tyrosine, threonine, and serine residues in the presence of [γ-^{32}P] ATP. Although it is not possible to estimate the absolute values of the relative rates of phosphorylation of each amino acid, since the rate of phospho amino acid destruction during acid hydrlysis differs considerably (Copper & Hunter, 1981) and the transfer of hydrolyzed samples is not quantitative, it is possible to evaluate the relative effects of inhibitors and activators by comparing the ratios of phospho amino acids that are released from the protein under identical conditions. It is apparent from Table IV that EGF stimulated overall phosporylation about 2–3-fold but tyrosine phosphorylation was stimulated over 15-fold. Moreover, there also appears to be a small stimulation of threonine phosphorylation. Particularly striking is the specific effect of Boc-Leu-CH_2Br. Whereas a pronounced inhibition of tyrosine phosphorylation was seen, neither threonine nor serine phosphorylation was inhibited in the absence of EGF. In the presence of EGF the inhibition of tyrosine phosphorylation was even more pronounced and now some inhibition of threonine and serine phosphorylation was also noted. Further experiments are needed to establish whether these secondary effects of EGF are reproducible and significant.

Table IV: Inhibition of Tyrosine Phosphorylation by Boc-Leu-CH_2Br[a]

P-amino acid	cpm –EGF	–EGF, +Boc-Leu-CH_2Br	+EGF	+EGF, +Boc-Leu-CH_2Br
P-tyrosine	1152	488	18 000	5554
P-threonine	1100	1180	1 994	1270
P-serine	328	300	494	262
total phosphoprotein	3408	2024	12 204	5332

[a] The experimental conditions were the same as described in Figure 8. The radioactivity on the paper was measured by excising the radioactive spots corresponding to the phospho amino acid markers. After the ninhydrin color of the paper spots was bleached with 6% hydrogen peroxide, the radioactivity was quantified in a liquid scintillation counter. The total phosphoprotein of the reaction mixture was determined as described under Experimental Procedures and these values represent an aliquot of 10 μL of the total reaction mixture (70 μL). The final concentration of Boc-Leu-CH_2Br was 349 nmol/mg of protein.

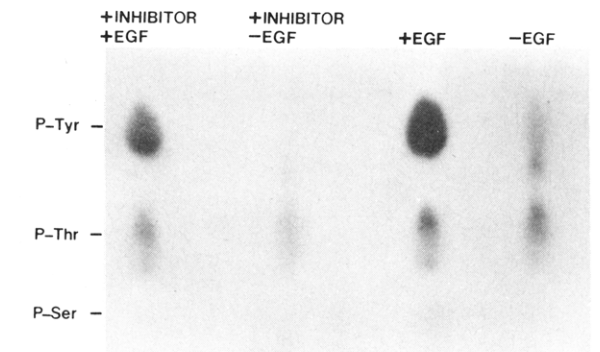

FIGURE 8: Inhibition by Boc-Leu-CH_2Br of tyrosine phosphorylation. Triton extracts of A-431 membranes (37.5 μg of protein) were labeled with [γ-^{32}P]ATP (5 μM, 5 μCi) at 0 °C for 3 min in the presence and absence of Boc-Leu-CH_2Cl (349 nmol/mg of protein). The proteins were hydrolyzed and subjected to high-voltage paper electrophoresis as described under Experimental Procedures. Phosphotyrosine, phosphothreonine, and phosphoserine were used as markers.

Effect of Boc-Leu-CH_2Br on Protein Phosphorylations Catalyzed by pp60^{src} and pl30^{fps} Tyrosine Kinases and Placental Membranes. In view of the rather specific inhibition by Boc-Leu-CH_2Br of tyrosine phosphorylation, the effect of the inhibitor was tested on two other tyrosine specific protein kinases, pp60 and pl30^{fps} encoded by the transforming genes of Rous and Fujinami sarcoma viruses, respectively. As shown in Table V, experiment 1, the background radioactivity (presumably caused by the *Staphylococcus* suspensions) was relatively high but unaffected by the inhibitor. The phosphorylation of the immune complex catalyzed by pp60^{src} and

Table V: Phosphorylation by Immunoprecipitates Obtained from Rous and Fujinami Sarcoma Virus Transformed Cells[a]

kinase system	immune complex phosphorylation (cpm in *Staphylococcus* suspension)		casein phosphorylation (cpm in supernatant)	
	no inhibitor	Boc-Leu-CH_2Br	no inhibitor	Boc-Leu-CH_2Br
experiment 1				
$pp60^{src}$ + control serum	7872	7516		
$pp60^{src}$ + TBR serum	12984 (5112)	10718 (3202)		
$pl30^{fps}$ + control sera	9140	8898		
$pl30^{fps}$ + TBR serum	29244 (20104)	15222 (6324)		
experiment 2				
$pl30^{fps}$ + control serum	1184	1286	326	226
$pl30^{fps}$ + TBR serum	7663	1656	4442	618

[a] The reaction mixture for the first experiment contained 20 mM Hepes, pH 7.4, 1 mM $MnCl_2$, immunoprecipitates of Rous sarcoma virus transformed cell extracts with tumor-bearing rabbit serum (TBR), and *Staphylococcus* protein A suspension (100 μg of protein) or immunoprecipitates of Fujinami sarcoma virus transformed cell extracts with anti-gag serum and *Staphylococcus* protein A suspension (100 μg of total protein) in a final volume of 70 μL. The reaction was initiated by addition of [γ-^{32}P]ATP (1 μM, 4 μCi). After 10 min at room temperature the reaction was terminated by pipetting 20-μL aliquots onto 2 × 2 cm squares of Whatman 3MM filter paper that were dropped into 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. The final concentration of Boc-Leu-CH_2Br was 250 nmol/mg of protein. The samples labeled $pp60^{src}$ and $pl30^{fps}$ were controls exposed to normal serum instead of TBR serum. Values in parentheses represent the specific phosphorylation induced by sarcoma virus infected cells. The reaction mixture for the second experiment was essentially the same except that casein (1 mg/mL) was added to the incubation. Furthermore, the reaction was terminated by addition of an excess of unlabeled ATP (2.5 mM) and immediately cooled at 0 °C. Phosphorylated casein was separated from the immunoprecipitate by centrifugation at 3000*g* for 3 min. The pellet was resuspended in 70 μL of 10 mM Hepes, pH 7.5, and 20-μL aliquots were taken for quantification of the radioactivity. The amount of casein phosphorylated in the supernatant was determined by pipetting 20-μL aliquots onto 2 × 2 cm squares of Whatman 3MM filter paper and proceeding as described above.

by $pl30^{fps}$ kinases was inhibited by Boc-Leu-CH_2Br, with Fujinami protein kinase being more sensitive than the Rous sarcoma virus induced protein kinase. With a partially purified preparation of Fujinami protein kinase immune complex the background radioactivity was reduced and the inhibition by Boc-Leu-CH_2Br was more striking (Table V, experiment 2). Furthermore, the phosphorylation of casein catalyzed by the Fujinami protein kinase was also strongly inhibited by Boc-Leu-CH_2Br and the background counts were virtually eliminated. In both cases the concentration of inhibitor required for a significant inhibition was of the same order of magnitude that inhibited extracts of plasma membranes of A-431 cells (250 nmol/mg of protein).

The phosphorylation mediated by $pl30^{fps}$ kinase in the presence of cysteine (20 mM) or dithiothreitol (50 mM) was enhanced 2-fold. The effect of Boc-Leu-CH_2Br depended on the order of addition of SH reagent and inhibitor. Incubation of the immunoprecipitate with an SH reagent followed by addition of Boc-Leu-CH_2Br did not inhibit phosphorylation. On the other hand, incubation of the inhibitor with the immunoprecipitate followed by addition of an SH reagent resulted in a very dramatic inhibition (data not shown). These findings suggest that Boc-Leu-CH_2Br acts as an irreversible inhibitor like iLac-CH_2Cl as reported by Johnson et al. (1982). Maximal inhibition was observed when Boc-Leu-CH_2Br was incubated with the kinase system for at least 10 min at room temperature prior to the addition of ATP.

It was shown previously (Carpenter et al., 1980) that plasma membrane from placenta catalyzes EGF-stimulated phosphorylation; however, significant inhibition of phosphorylation in Triton extracts of placental plasma membrane required 2–5 times higher concentrations of inhibitor than extract from A-431 cells. $NaDodSO_4$–polyacrylamide gel electrophoresis have revealed that Boc-Leu-CH_2Br inhibited preferentially the phosphorylation of EGF receptor (data not shown). These findings and the observation that iLac-CH_2Cl did not inhibit cAMP-dependent protein kinase (Johnson et al., 1982) suggest that these halomethyl ketone derivatives may under appropriate conditions be used as specific inhibitors of tyrosine phosphorylating kinases.

We conclude from these observations that halomethyl ketone derivatives may be useful tools in probing various protein kinase activities of cells. The preferential inhibition of EGF-stimulated phosphorylation of tyrosine residues is of particular interest in view of the established protein kinase activity of transforming gene products of several tumor viruses. We have therefore initiated experiments to explore the effect of halomethyl ketones on the growth of normal and transformed cells. We also hope to survey additional halomethyl ketone derivatives in the hope of finding inhibitors for threonine- and serine-specific protein kinases as well.

Acknowledgments

We thank Dr. Volker Vogt and William Potts for providing us with the immunocomplexes prepared from Rous and Fujinami virus transformed cell lines. We are grateful to Dr. Charles Kettner of Du Pont and to Dr. James Powers of Georgia Institute of Technology for samples of halomethyl ketone derivatives. A sample of the synthetic chloromethyl ketone derivative of lactic acid was also prepared for us at the National Cancer Institute through the courtesy of Dr. M. B. Naff. Most generous gifts of [γ-^{32}P]-8-azido ATP were received from Dr. B. E. Haley of the University of Wyoming.

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Activation of Magnesium Ion Specific Adenosinetriphosphatase in Chloroplast Coupling Factor 1 by Octyl Glucoside†

Uri Pick* and Sara Bassilian

ABSTRACT: The effects of the neutral detergent octyl glucoside on ATPase activity of chloroplast coupling factor 1 (CF_1) have been studied. (1) The presence of octyl glucoside above its critical micellar concentration activates Mg-ATPase but not Ca-ATPase of CF_1. The optimal detergent concentration for activation at 37 °C is 30 mM and the maximal rate of ATP hydrolysis is 20-40 μmol (mg of protein)$^{-1}$ min^{-1}. Conversely, a brief preincubation of CF_1 with 30 mM octyl glucoside in the presence of ATP, followed by a dilution of the detergent, activates Ca-ATPase but not Mg-ATPase of CF_1. (2) Mg-ATPase activation is highly cooperative with respect to octyl glucoside concentration. A similar Hill *n* value of about 7.5 was calculated for both the octyl glucoside monomers and for the detergent micelles. (3) The incubation of CF_1 with the detergent at 37 °C in a buffer solution causes an irreversible inactivation of the ATPase activity. ATP, ADP, and high concentrations of salt effectively protect against inactivation, but inorganic phosphate, dithiothreitol (DTT), and 5′-adenylyl imidodiphosphate [AMP-P(NH)P] are completely ineffective. (4) The addition of 30 mM octyl glucoside to CF_1 preparations that had been preactivated by trypsin, heat, DTT, or octyl glucoside stimulates Mg-ATPase and inhibits Ca-ATPase of CF_1, suggesting a reversible modification of the catalytical properties of CF_1 by the detergent. (5) Of the detergents tested only two classes, the bile detergents and saturated fatty acids containing an 8-12 carbon chain, slightly activate CF_1-Mg-ATPase at above their critical micellar concentration. These results suggest that stimulation of CF_1-Mg-ATPase is obtained by its interaction with small detergent micelles. (6) In the presence of octyl glucoside, free Mg^{2+} appears to be a partial noncompetitive inhibitor with respect to the substrate and only partly inhibits ATPase activity even at 10-100-fold excess over the substrate, Mg-ATP. $K_{is}(Mg^{2+})$ = 0.9 mM, $K_{ii}(Mg^{2+})$ = 10 mM, K_s(Mg-ATP) = 150 μM, and V = 40 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}. Free ATP is a competitive inhibitor with respect to the substrate. K_i(ATP) = 2.6 mM, K_s(Mg-ATP) = 60 μM, and V = 31 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}. The results suggest that the high apparent Mg-ATPase activity that is obtained in the presence of octyl glucoside is mainly due to the decreased inhibition by free Mg^{2+} ions and to the increase in the affinity for the substrate Mg-ATP. (7) The mechanism of CF_1-ATPase activation by octyl glucoside and its possible physiological implications are discussed.

The chloroplast coupling factor (CF_1)[1] is part of the DCCD-sensitive ATPase complex that phosphorylates ADP in the process of photophosphorylation (Avron, 1963; Vambutas & Racker, 1965). In broken thylakoid preparations the enzyme does not hydrolyze ATP unless it is preactivated by illumination in the presence of dithiol reagents (Petrack & Lipmann, 1961). The activated enzyme hydrolyzes ATP in the presence of Mg^{2+} but not Ca^{2+} ions. ATP hydrolysis is coupled to proton uptake into the thylakoid membrane and probably reflects the reversal of ATP synthesis (Carmeli, 1970). It has also been recently demonstrated that illumination of intact chloroplasts can activate the CF_1-ATPase in the absence of exogenous dithiol reagents (Mills & Hind, 1979). It appears, therefore, that light activation of the ATPase occurs also in vivo.

ATPase activity of the solubilized CF_1 is also very poor, but it can be activated by trypsin (Vambutas & Racker, 1965;

† From the Biochemistry Department, The Weizmann Institute of Science, Rehovot 76100, Israel. *Received May 7, 1982.* This research was supported by a grant from the U.S.-Israel Agricultural Research and Development Fund (BARD, Grant I-107-79).

[1] Abbreviations: CF_1, chloroplast coupling factor 1; OG, octyl β-D-glucopyranoside; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; AMP-P(NH)P, 5′-adenylyl imidodiphosphate; quercetin, 3,3′,4′,5,7-pentahydroxyflavone; phloridzin, 1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone; cmc, critical micellar concentration; DCCD, dicyclohexylcarbodiimide; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; FITC, fluorescein 5′-isothiocyanate; PMS, *N*-methylphenazonium methosulfate; ANS, 8-anilino-1-naphthalenesulfonate; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-6144$01.25/0