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Genistein, a Specific Inhibitor of Tyrosine-specific Protein Kinases*

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Tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor, pp60$^{v\text{-}src}$ and pp110$^{gag\text{-}fes}$ was inhibited *in vitro* by an isoflavone genistein. The inhibition was competitive with respect to ATP and noncompetitive to a phosphate acceptor, histone H2B. By contrast, genistein scarcely inhibited the enzyme activities of serine- and threonine-specific protein kinases such as cAMP-dependent protein kinase, phosphorylase kinase, and the Ca^{2+}/phospholipid-dependent enzyme protein kinase C. When the effect of genistein on the phosphorylation of the EGF receptor was examined in cultured A431 cells, EGF-stimulated serine, threonine, and tyrosine phosphorylation was decreased. Phosphoamino acid analysis of total cell proteins revealed that genistein inhibited the EGF-stimulated increase in phosphotyrosine level in A431 cells.

Tyrosine-specific protein kinase activity is known to be associated with oncogene products of the retroviral *src* gene family (1–3). This kinase activity is strongly correlated with the ability of retroviruses to transform cells, since mutants with reduced kinase activity have lower transforming efficiency, and mutants which lack tyrosine kinase activity are transformation-defective (4). Similar kinase activity is also associated with the cellular receptors for several growth factors such as EGF[1] (5), platelet-derived growth factor (6, 7), insulin (8, 9), and insulin-like growth factor I (10, 11). Therefore, it is possible that tyrosine pohosphorylation plays an important role for cell proliferation and cell transformation.

According to this hypothesis, a specific inhibitor for tyrosine kinases could be an antitumor agent as well as a tool for understanding the physiological role of tyrosine phosphorylation. Although not so specific for tyrosine kinases, several compounds have been reported to inhibit tyrosine kinase activity. A protease inhibitor N^{β}-tosyl-L-lysyl chloromethyl ketone was demonstrated to inhibit tyrosine kinase activity associated with pp60$^{v\text{-}src}$ and revert the effects of avian sarcoma virus transformation on cell morphology, adhesion, and glucose transport (12). A flavone quercetin was reported to inhibit the tyrosine kinase activity of pp60$^{v\text{-}src}$ (13, 14) as well as the activities of cAMP-independent protein kinase (15), the Ca^{2+}/phospholipid-dependent enzyme protein kinase C (16), phosphorylase kinase (17), Na^+,K^+-ATPase (18), and Ca^{2+},Mg^{2+}-ATPase (19). More recently, amiloride, which is well known as an inhibitor for Na^+,K^+ antiporter (20–22), was shown to directly inhibit growth factor receptor tyrosine kinase activity (23).

In the search for specific inhibitors for tyrosine kinases, we have recently isolated an isoflavone compound genistein from fermentation broth of *Pseudomonas* sp. (24). In this study, we show that genistein is a highly specific inhibitor for tyrosine kinases but scarcely inhibits the activity of serine and threonine kinases and other ATP analogue-related enzymes *in vitro*. Furthermore, genistein was revealed to inhibit EGF-stimulated phosphorylation in cultured A431 cells.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

In this study, we demonstrated that genistein inhibits the activities of tyrosine-specific protein kinases. Kinetic analysis revealed that inhibition of the EGF receptor kinase activity was competitive with ATP and that genistein leads to the formation of nonproductive enzyme-substrate complexes. Therefore, since Erneux *et al.* (39) have proposed that the reaction mechanism of the EGF receptor kinase is a sequential Ordered Bi Bi reaction with a peptide as the first substrate and ATP as the second, genistein could be expected to act uncompetitively with respect to a phosphate acceptor, histone H2B, *i.e.* genistein could bind to the enzyme only after histone combined (40). However, our results indicated that genistein was a noncompetitive inhibitor with respect to histone H2B. Since genistein bears no structural relationship to ATP, inhibition of the EGF receptor kinase activity by genistein may

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[1] The abbreviations used are: EGF, epidermal growth factor; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid (Miniprint); BSA, bovine serum albumin; RSV, Rous sarcoma virus; GA-FeSV, Gardner-Arnstein feline sarcoma virus; RIPA, radioimmune precipitation assay; NP-40, Nonidet P-40 (Miniprint).

[2] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–4, and Tables I–V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0280, cite the authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

not be due to true competition for exactly the same site as that utilized by ATP. Thus, it would be possible that genistein binds in multiple places in the reaction pathway and, consequently, appears noncompetitive with respect to a phosphate acceptor. In this regard, it is intriguing that quercetin, which has a structure closely related to genistein, has also been reported to be competitive with ATP and noncompetitive with respect to histone (14), whereas amiloride, which resembles the structures of purines and pyrimidines, is competitive with ATP and uncompetitive with histone (23).

Genistein exhibited specific inhibitory activity against tyrosine kinases, that is, the EGF receptor kinase and $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ kinases, but scarcely inhibited the activity of serine- and threonine-specific kinases such as cAMP-dependent protein kinase, protein kinase C, and phosphorylase kinase. These results are consistent with the fact that primary amino acid sequences of tyrosine kinases are closely related to each other but weakly homologous with the sequence of the catalytic subunit of cAMP-dependent protein kinase (1). Thus, genistein is not a mere ATP analogue, and can discriminate the differences in the catalytic site for ATP of these protein kinases. In addition, the activities of 5′-nucleotidase and phosphodiesterase were also poorly inhibited by genistein. The property of genistein to specifically inhibit tyrosine kinase activity is clearly different from that of a flavone quercetin, which has been reported to inhibit not only the tyrosine kinase activity associated with $pp60^{v\text{-}src}$ (13, 14) but also the activities of protein kinase C (16), phosphorylase kinase (17), Na^+,K^+-ATPase (18), and 5′-nucleotidase (Table I, Miniprint). High specificity of genistein will be advantageous for utilizing this compound as a tool for elucidating the role of tyrosine phosphorylation in cells.

When the effect of genistein on the phosphorylation of the EGF receptor was examined in cultured A431 cells, EGF-stimulated increase of tyrosine phosphorylation was observed to decrease. EGF-induced increase in the level of cellular phosphotyrosine was also inhibited by the treatment of A431 cells with genistein. These results indicate that genistein inhibits the tyrosine kinase activity of the EGF receptor in intact A431 cells. Furthermore, genistein was found to inhibit EGF-stimulated serine and threonine phosphorylation of the EGF receptor. This may result from *in vivo* direct inhibition of serine and threonine kinase activity which is responsible for phosphorylation of the EGF receptor. Alternatively, inhibition of the EGF receptor-associated tyrosine kinase activity may block a putative cellular pathway regulating serine and threonine kinase activity that phosphorylates the EGF receptor. Precise mechanism of this effect is, however, not known at the present time and remains to be determined.

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SUPPLEMENTAL MATERIAL TO:

GENISTEIN, A SPECIFIC INHIBITOR OF TYROSINE-SPECIFIC PROTEIN KINASES

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EXPERIMENTAL PROCEDURES

Materials --- Acacetin, prunetin, genistin and daidzein were purchased from Laboratories Sarget. Biochanin A was obtained from Aldrich, flavone, kaempferol and apigenin from Sigma and quercetin from Nakarai. Genistein was prepared from fermentation broth of Pseudomonas sp. as described previously (24).

Antibodies --- Mouse monoclonal anti-EGF receptor antibody (528 IgG) (25) was a gift of Dr. Tomoyuki Kawamoto (Okayama University). Sera from rabbits bearing tumors induced by Rous sarcoma virus (TBR sera) were kindly provided by Dr. Ryotaro Ishizaki (Nihon Medical School) (26). Anti-ppl10$^{gag-fes}$ antisera were obtained from Fisher rats bearing GA-FeSV-transformed 3Y1 cells.

Cells --- Human epidermoid carcinoma A431 cells (27) and RSV-transformed rat 3Y1 cells (28) were obtained from Drs. George J. Todaro (Washington University) and Reiko Hirai (The Tokyo Metropolitan Institute of Medical Science), respectively. GA-FeSV-transformed cells were prepared by transfecting 3Y1 cells with pBR322-GA-FeSV-proviral DNA recombinant plasmid which was obtained from Dr. Charles J. Sherr (National Cancer Institute) (29). Rat 3Y1 is the normal cell line established from Fisher rat kidney (30). These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal calf serum.

Enzymes --- The EGF receptor was purified from Triton X-100-solubilized membranes of A431 cells by sequential affinity chromatography on wheat germ agglutinin-agarose and tyrosine-Sepharose columns as described previously (31). The pp60^{v-src} kinase was purified from Triton X-100-solubilized membranes of Rous sarcoma virus-transformed chick embryo fibroblasts by sequential affinity chromatography on casein-agarose and tyrosine-Sepharose columns (32). Protein kinase C was purified from rabbit kidney by sequential chromatography on DEAE-Sepharose 4B and polyacrylamide-immobilized phosphatidylserine according to Uchida et al. (33). cAMP-dependent protein kinase (rabbit muscle), phosphorylase kinase (rabbit muscle), phosphodiesterase (bovine heart) and 5'-nucleotidase (snake venom) were obtained from Sigma.

Assay of enzyme activity--- EGF receptor kinase reactions were performed in a final volume of 50 µl containing 20 mM Pipes- NaOH pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 100 µM sodium vanadate, 10 µM [γ-^{32}P]ATP (4 mCi/umol), 1 µg/ml mouse EGF (Collaborative Research), 10 µg of A431 cell membrane, which was prepared as described previously (31), and the inhibitor. When the kinase activity against an exogenous substrate was assayed, histone H2B (25 µg) (Sigma) was included in the reaction mixture. The reaction was continued for 5 min at 0°C and terminated by addition of Laemmli's SDS sample buffer (34) and by boiling for 2 min. The samples were analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography. The bands of the EGF receptor were excised from the gels and the radioactivity was counted with a liquid scintillation counter. In table I, the effect of genistein was examined by using the purified EGF receptor instead of A431 cell membrane; the purified EGF receptor (0.1 µg), an exogenous substrate, histone H2B (25 µg), and 0.05 % TX-100 were included in the reaction mixture described above. The reaction was performed at 30°C for 3 min. cAMP-dependent protein kinase activity was assayed in a reaction mixture (final volume of 50 µl) containing 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 10 µM [γ-^{32}P]ATP (2 mCi/pmol), 6 mg/ml histone type IIA (Sigma), 10 µM cAMP, 4 µg of the enzyme and the inhibitor. Phosphorylase kinase activity was assayed in a reaction mixture (final volume of 50 µl) consisting of 40 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 10 µM [γ-^{32}P]ATP (2 mCi/µmol), 10 µg of phosphorylase b (Sigma), 2 µg of the enzyme and the inhibitor. Activity of protein kinase C was assayed in a reaction mixture (final volume of 50 µl) containing 25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 µM [γ-^{32}P]ATP (4 mCi/µmol), 50 µg/ml of phosphatidylserine, 0.5 µg/ml of diolein, 0.5 mM $CaCl_2$, 10 µg of histone H1 (Sigma), 0.1 µg of the enzyme and the inhibitor. The reaction mixture was incubated for 3 min at 30°C and analyzed as described for EGF receptor kinase. Phosphodiesterase activity was assayed in a reaction mixture (final volume of 50 µl) composed of 50 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM DTT, 10 µM [^{14}C]cAMP (286 mCi/mmol), 3 ng of the enzyme and the inhibitor. 5'-Nucleotidase activity was assayed in a reaction mixture (final volume of 50 µl) containing 55 mM Tris-HCl pH8.5, 5.5 mM $MgCl_2$, 10 µM [^{14}C]AMP (58 mCi/mmol), 10 mM sodium potassium tartrate, 2 ng of the enzyme and the inhibitor. After incubating for 3 min at 30°C, the reaction was terminated by boiling for 45 sec. The reaction product was analyzed by thin-layer chromatography on cellulose in ethyl alcohol/ 1.0 M ammonium acetate pH 7.5. The spot of adenosine was scraped and the radioactivity was measured with a liquid scintillation counter. Tyrosine kinase activity of pp60^{v-src}, immunoprecipitated by TBR sera and protein A-Sepharose 4B from the detergent lysate of RSV transformed-3Y1 cells was assayed in a reaction mixture (final volume of 50 µl) containing 20 mM Pipes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 10 µM [γ-^{32}P]ATP (2 mCi/µmol). Autophosphorylation activity of pp110$^{gag-fes}$ immunoprecipitated from Feline sarcoma virus-transformed 3Y1 cells was assayed under similar conditions. After incubating for 5 min at 30°C, the reaction was stopped by the addition of Laemmli's SDS sample buffer and by boiling for 2 min. The reaction product was analyzed by SDS-polyacrylamide gel electrophoresis as described for the EGF receptor. In table I, purified pp60^{v-src} was also used to examine the effect of genistein; purified pp60^{v-src} (0.05 µg) and an exogenous substrate, casein (25 µg), were included in the reaction mixture. The reaction was performed at 30°C for 3 min and the reaction products were analyzed similarly.

Immunoprecipitation of the EGF receptor---A431 cells were labeled for 6 h in phosphate-free Dulbecco's modified Eagle's medium supplemented with 2 % dialyzed fetal calf serum and 1 mCi/ml of [^{32}P]orthophosphate (Amersham), and incubated with EGF (100 ng/ml) and the inhibitor for the last 15 min. The labeled cells were lysed in RIPA buffer (40 mM Hepes-NaOH pH7.4, 1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate, 0.15 M NaCl, 1 mM PMSF, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate) (35), and the lysates were incubated with anti-EGF receptor antibody (528 IgG) (25). The immunocomplexes were adsorbed to protein A-Sepharose 4B and washed extensively with RIPA buffer (36). The immunoprecipitates were analyzed on a SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Phosphoamino acid analysis--- The phosphorylated EGF receptor separated by SDS polyacrylamide gel electrophoresis was eluted from the gel and subjected to acid-hydrolysis in 6 N HCl for 1.5 h at 110 °C. The phosphoamino acids were resolved by two-dimensional separation of electrophoresis at pH 1.9 followed by electrophoresis at pH 3.5 (37). For the estimation of the level of phosphotyrosine in total cellular protein, cell protein was extracted with phenol from the detergent lysate of [^{32}P]phosphate-labeled A431 cells and then precipitated with 20 % TCA. The cell protein thus obtained was acid-hydrolyzed and subjected to two-dimensional separation as described above.

Binding of ^{125}I-EGF to A431 cells--- A431 cells were preincubated in serum-free medium for 1 h prior to adding ^{125}I-EGF (100 µCi/pg, Amersham). EGF binding was measured by incubating at 4°C for 1 h in the presence of 2 ng of labeled EGF in 1 ml of binding medium (DMEM, 5 mM Hepes pH 7.5, 0.1 % BSA). Nonspecific binding was measured with 2 µg/ml unlabeled EGF. After the incubation, the monolayers were washed four times with 2 ml of binding medium and solubilized with 1 ml of 1 N NaOH at 37 °C for 1 h (38).

RESULTS

Inhibition of tyrosine kinase activity by genistein --- Incubation of A431 cell membranes with [γ-^{32}P]ATP resulted in the autophosphorylation of the 170-kD EGF receptor (Fig. 1A, lane 1; Fig. 2A). Addition of genistein to the reaction mixture caused a dose-dependent inhibition of autophosphorylation of the EGF receptor (Fig. 1A, lane 2; Fig. 2A). The half maximal effect was observed at 0.7 µg/ml of genistein (Table II). When an exogenous substrate protein, histone H2B, was included in the reaction mixture, phosphorylation of histone H2B proceeded linearly for at least 3 min (Fig. 2B). This was also inhibited by genistein in a dose-dependent manner (Fig. 2B). The half maximal effect was observed at 5.5 µg/ml of genistein, which was considerably higher than that observed for the autophosphorylation. Similarly, phosphorylation of IgG by pp60^{v-src} and autophosphorylation of ppl10$^{gag-fes}$ were inhibited by genistein (Fig. 1B and 1C).

By contrast, genistein scarcely inhibited serine- and threonine-specific protein kinases such as cAMP-dependent-protein kinases, protein kinase C and phosphorylase kinase at 100 µg/ml (Table I). In addition, genistein did not show inhibitory activity against other ATP-analogue-related enzymes such as phosphodiesterase and 5'-nucleotidase (Table I). Thus, inhibitory activity of genistein was highly specific for tyrosine protein kinases. On the other hand, a flavone quercetin inhibited not only tyrosine kinases but also protein kinase C, phosphorylase kinase and 5'-nucleotidase (Table I).

Effect of genistein on the kinetics of the EGF receptor kinase activity --- Fig. 3A shows the effect of various concentrations of ATP on genistein-mediated inhibition of the EGF receptor kinase activity against histone H2B. Genistein was a competitive inhibitor for the EGF receptor-catalyzed phosphorylation of histone H2B. The K_m value for ATP of the EGF receptor kinase was 3.6 µM and the K_i was 3.7 ug/ml. When the concentration of histone H2B was varied, a different kind of inhibition pattern was observed. As shown in Fig. 3B, genistein acted as a non-competitive inhibitor with respect to histone H2B as a substrate.

Effect of isoflavones and related compounds on the EGF receptor kinase activity --- To investigate structure-activity relationships, effect of several isoflavones and related compounds on the EGF receptor kinase activity was examined (Table II). Among isoflavones, prunetin which has a methyl group in position 7 exhibited rather strong activity, while the introduction of a glucose residue at this position (genistin) abolished the activity. This may be due to a steric hindrance by a bulky sugar group. The introduction of a methoxy group at 4' position (biochanin A) lowered the inhibitory activity. Interestingly, daidzein which is devoid of hydroxyl group in position 5 did not show any inhibitory activity at 100 µg/ml. Flavones such as apigenin, acacetin and flavone exhibited only low activity. Flavonol such as kaempferol and quercetin was highly active.

Effect of genistein on the phosphorylation of the EGF receptor in vivo. --- We next examined the effect of genistein on the EGF-stimulated phosphorylation of the EGF receptor in intact cells. A431 cells were labeled with [^{32}P]orthophosphate for 6 h and incubated with EGF for the last 15 min in the presence of various concentrations of genistein. The phosphorylated EGF receptor was immunoprecipitated and analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography. As shown in Fig. 4, the phosphorylation of the EGF receptor decreased in the presence of genistein. The half maximal effect of genistein was observed at 30 µg/ml. Phosphoamino acid analysis of the EGF receptor (Table III) showed that genistein decreased the phosphorylation of the receptor on serine- and threonine residues as well as that on tyrosine residues. Thus, in intact cells, genistein inhibited not only the autophosphorylation of the EGF receptor but also the phosphorylation of the receptor by other serine- and threonine-specific kinases.

Effect of genistein on the EGF receptor kinase activity in vivo --- To investigate the effect of genistein on the EGF receptor kinase activity in intact cells, the level of phosphotyrosine in total cellular protein phosphate was estimated. Cellular proteins were extracted from A431 cells prelabeled with [^{32}P]orthophosphate and subjected to phosphoamino acid analysis. Table IV shows that treatment of the cells with genistein blocked the effect of EGF to increase the level of phosphotyrosine. This result indicates that genistein also inhibits the tyrosine-specific kinase activity associated with the EGF receptor in intact A431 cells.

Effect of genistein on the binding of ^{125}I-EGF to A431 cells --- A431 cells were incubated with ^{125}I-EGF for 1 h in the presence of various concentrations of genistein and the radioactivity of ^{125}I-EGF bound to A431 cells was counted. As indicated in Table V, genistein scarcely inhibited ^{125}I-EGF binding to A431 cells.

TABLE I

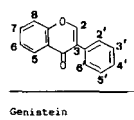
Effect of genistein on the enzyme activities.

Enzymes	Genistein	Quercetin
	ID_{50} µg/ml	
EGF receptor	6.0[a]	8.0[a]
pp60^{v-src}	7.0[a]	8.0[a]
	8.0[b]	-
ppl10$^{gag-fes}$	6.5[c]	-
cAMP-dependent protein kinase	> 100	>100
Protein kinase C	> 100	25
Phosphorylase kinase	> 100	5.0
5'-nucleotidase	> 100	30
Phosphodiesterase	> 100	-

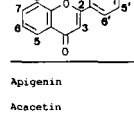
The enzyme activities were measured at 30°C for 3 min as described under "Experimental Procedures" in the absence or presence of various concentrations of genistein (1.0, 2.5, 5.0, 10, 25, 50, 100 µg/ml). The concentration for 50% inhibition was determined from the inhibition curve taking probit of inhibition percent at varied concentrations of genistein on ordinate and logarithm of concentrations of genistein on abscissa (41, 42). [a]Purified EGF receptor (0.1 µg) and pp60^{v-src} (0.05 µg) were used to assay the phosphorylation of exogenous substrates (histone H2B (25 µg) and casein (25 µg), respectively). [b]Phosphorylation of bound IgG by immunoprecipitated pp60^{v-src} was assayed [c]Autophosphorylation of immunoprecipitated pl10$^{gag-fes}$ was assayed.

TABLE II

Effect of genistein and related compounds on the EGF receptor kinase and cAMP-dependent kinase activities



	5	7	4'	ID_{50} µg/ml EGF receptor[a] kinase	cAMP-dependent protein kinase
Genistein	OH	OH	OH	0.7	> 100
Genistin	OH	O-glucose	OH	> 100	-
Prunetin	OH	OCH_3	OCH_3	4.2	-
Daidzein	-	OH	OH	> 100	-
Biochanin A	OH	OH	OCH_3	26.0	> 100



	3	5	7	3'	4'			
Apigenin	-	OH	OH	-	OH	25.0	> 100
Acacetin	-	OH	OH	-	OCH_3	40.0	-
Flavon	-	-	-	-	-	50.0	> 100
Kaempferol	OH	OH	OH	-	OH	3.2	> 100
Quercetin	OH	OH	OH	OH	OH	5.0	> 100

[a]Autophosphorylation of the EGF receptor kinase was assayed by incubating A431 cell membranes with [γ-^{32}P]ATP in the presence of EGF (1 µg/ml) at 0°C as described under "Experimental Procedures".

TABLE III

Phosphoamino acid analysis of the EGF receptor of A431 cells treated with genistein

	Total	Phospho-tyrosine	Phospho-threonine	Phospho-serine
	%			
Control	100	0.4	32	68
EGF	310	11.0	81	219
EGF + Genistein	146	1.2	45	100

A431 cells were labeled for 6 h with [^{32}P]phosphate and then treated with and without EGF (100 ng/ml) for 15 min in the absence or presence of genistein (40 µg/ml). The EGF receptor was immunoprecipitated and analyzed as described in the legend to Fig. 4. The phosphorylated EGF receptor was eluted from the gel and subjected to acid hydrolysis in 6 N HCl for 1.5 h at 110°C. The phosphoamino acids were separated by two-dimensional thin layer electrophoresis. Spots corresponding to phosphoserine, phosphothreonine, and phosphotyrosine were identified with ninhydrin and cut out from thin layer plates. Radioactivity of each phosphoamino acid was counted and the results are presented as percent of total phosphoamino acids detected in the EGF receptor derived from untreated control cells. 100% represents 806 cpm.

TABLE IV

Phosphoamino acid analysis of the total cellular proteins of A431 cells eated with genistein

	Total	Phospho-tyrosine	Phospho-threonine	Phospho-serine
Control	100	0.09	5.7	94
EGF	106	0.57	6.7	99
EGF + Genistein	94	0.16	5.5	88

A431 cells were labeled for 6 h with [^{32}P]phosphate and then treated with or without EGF (100 ng/ml) for 15 min in the absence or presence of genistein (40 μg/ml). The cell proteins extracted with phenol and precipitated with 20 % TCA were acid-hydrolyzed and subjected to two-dimensional separation by electrophoresis at pH 1.9 and pH 3.5. Spots corresponding to phosphoserine, phosphothreonine, and phosphotyrosine were identified with ninhydrin and cut out from the thin layer plates. Radioactivity of each phosphoamino acid was counted and the results are presented as percent of total phosphoamino acids in untreated control cells. 100% represents 756,000 cpm.

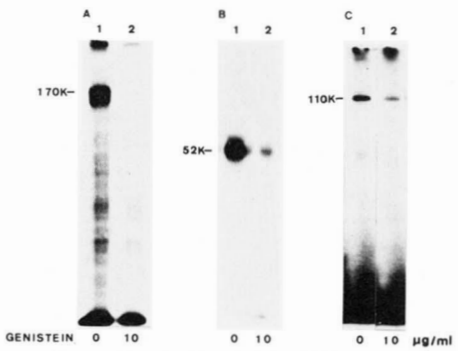


Fig. 1. Effect of genistein on the tyrosine kinase activity of the EGF receptor, pp60$^{v\text{-}src}$ and pp110$^{gag\text{-}fes}$. A431 cell membranes (A) were incubated with EGF (1 μg/ml) and [γ-^{32}P] ATP in the absence (lane 1) or presence of 10 μg/ml genistein (lane 2) for 5 min at 0 °C. The reaction products were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography as described under "Experimental Procedures". Tyrosine kinase assay of pp60$^{v\text{-}src}$ immunoprecipitated from RSV-transformed 3Y1 cells (B) and pp110$^{gag\text{-}fes}$ immunoprecipitated from Feline sarcoma virus-transformed 3Y1 cells (C) were carried out at 25°C for 5 min and analyzed similarly.

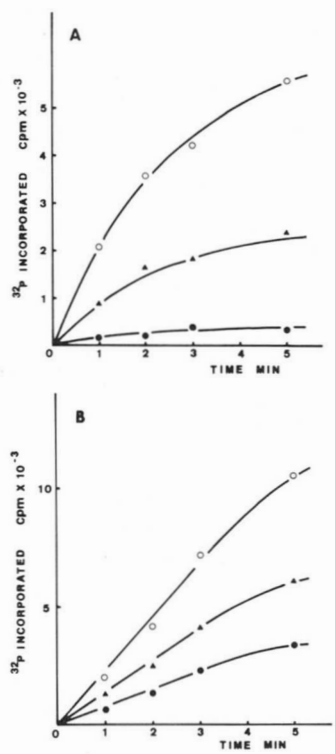
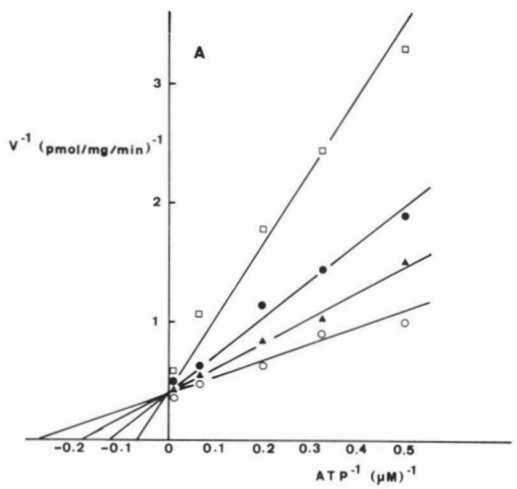


Fig. 2 Effect of genistein on the time course of autophosphorylation of the EGF receptor (A) and phosphorylation of histone H2B by the EGF receptor kinase (B). A431 cell membranes were incubated with EGF (1 μg/ml), [γ-^{32}P]ATP (40 μM) and sodium vanadate (100 μM) in the absence (○) or presence of genistein (A: ▲, 1 μg/ml; ●, 10 μg/ml; B: ▲, 5 μg/ml; ●, 10 ug/ml) for the indicated time periods at 0°C. In panel (B), histone H2B (500 μg/ml) was included in the reaction mixture. The reaction products were analyzed by SDS polyacrylamide gel electrophoresis and the bands of the EGF receptor (A) or histone H2B (B) were excised from the gels and the radioactivity was counted with a liquid scintillation counter as described under "Experimental Procedures".

TABLE V

Effect of genistein on ^{125}I-EGF binding to A431 cells.

Genistein μg/ml	^{125}I-EGF bound cpm
0	37483
10	36410
40	37077
100	30475



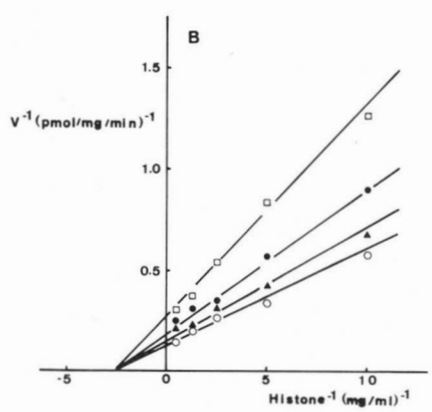


Fig. 3 Effect of genistein on the kinetics of the EGF receptor kinase activity. (A) A431 cell membranes were incubated with EGF (1 μg/ml), sodium vanadate (100 μM), histone H2B (200 μg/ml) and various concentrations of [γ-^{32}P]ATP in the absence (○) or presence of genistein (▲, 1 μg/ml; ●, 3 μg/ml; □, 10 μg/ml) for 3 min at 0°C. The reaction products were analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography. The bands of histone H2B were excised from the gels and the radioactivity was counted with a liquid scintillation counter. (B) The EGF receptor kinase activity was assayed with various concentrations of histone H2B in the absence (○) or presence of genistein (▲, 1 μg/ml; ●, 3 μg/ml; □, 10 μg/ml) for 3 min at 0°C. The concentration of [γ-^{32}P]ATP was 40 μM.

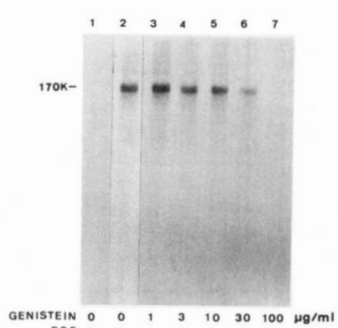


Fig. 4 Effect of genistein on the phosphorylation of the EGF receptor in vivo. A431 cells were labeled for 6 h with [^{32}P]phosphate and then EGF (100 ng/ml) and various concentrations of genistein were added for the last 15 min. The detergent lysates of the labeled cells were immunoprecipitated with anti-EGF receptor antibody and protein A-Sepharose. The immunoprecipitates were then analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography.