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Specific Desensitization of the Epidermal Growth Factor Receptor by pp60$^{v\text{-}src}$

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BALB/c 3T3 cells infected with a temperature-sensitive mutant (LA90) of RSV have been used to investigate possible heterologous interactions between the pp60$^{v\text{-}src}$ tyrosyl kinase and the epidermal growth factor (EGF) and bradykinin receptors. The LA90 pp60$^{v\text{-}src}$ exhibits a very rapid activation $t_{1/2}$ (<5 min) of protein kinase activity on decreasing the temperature from 40°C to 35°C. This change in temperature was also found to induce a very rapid decrease in the affinity for ^{125}I-EGF of receptors on the RSV-LA90-infected cells but not of those on control parental cells. However, no significant changes were detected in the binding of ^{3}H-bradykinin to either cell line. Two separable processes control the desensitization of the EGF receptor by pp60$^{v\text{-}src}$, both of which are independent of protein kinase C. The first is rapid and transient, while the second is sensitive to cycloheximide and persists long after inactivation of pp60$^{v\text{-}src}$.

Key words: oncogene, protein kinase C, tyrosine kinase

The oncogene product of the Rous sarcoma virus, pp60$^{v\text{-}src}$, and its cellular homolog pp60$^{c\text{-}src}$, are 60-kilodalton, membrane-associated proteins, with tyrosyl-specific protein kinase activity [1] similar to that exhibited by a number of growth factor receptors, including those for epidermal growth factor (EGF) [2] and platelet-derived growth factor (PDGF) [3]. Activation of pp60$^{v\text{-}src}$ has been reported to stimulate phosphatidylinositol (PI) turnover [4], and NIH 3T3 cells transformed by v-*src*, or by the oncogene v-*ras* [6], possess elevated levels of diacylglycerol, an intracellular second messenger generated by PI turnover, that activates the calcium- and phospholipid-dependent protein kinase C (PKC) [5,6]. V-*src*- and v-*ras*-infected NIH 3T3 cell lines also exhibit increased PKC activity, relative to uninfected parental cells, as estimated

Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified eagle's medium; EGF, epidermal growth factor; HEPES 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate, 13-acetate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGF, transforming growth factor; ts-, temperature sensitive.

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from the basal level of phosphorylation of an endogenous 80-kD PKC substrate [5,6]. PKC can decrease the affinity of the EGF receptor for EGF by phosphorylating residue Thr 654 of the EGF receptor [e.g., 2,7]. To investigate further the possible role of PKC in the phenotypic changes induced by oncogene products, we examined the effect of $pp60^{v\text{-}src}$ on EGF binding. The binding of bradykinin was also studied, because the expression of the v-*ras* oncogene appears to cause a dramatic increase in the receptor concentration for this peptide [8]. Although early studies documented a loss of EGF binding to cells transformed by a variety of different oncogenes, including v-src, [e.g., 9] and the constitutive activity of $pp60^{v\text{-}src}$ has been reported to down-regulate expression of the EGF receptor [10], acute heterologous interactions had not previously been investigated.

MATERIALS AND METHODS

Materials

EGF was from Collaborative Research (Bedford, MA). [^{125}I]NaI was obtained from Amersham Corp. [^{3}H]-bradykinin was from NEN. PMA was from LC Systems (Woburn, MA). All other reagents were from Sigma (St. Louis, MO).

Cell Lines

The temperature-sensitive Rous sarcoma virus mutant RSV-LA90 (Schmidt-Ruppin group D) was generated and initially characterized by Peter Vogt (University of California, Los Angeles) and used to transform BALB/c mouse embryo fibroblasts by Joan Brugge (Stony Brook, NY). All cultures were grown in Dulbecco's modified Eagle's medium with 10% calf serum (Hyclone, Logan, UT) plus penicillin/streptomycin.

EGF Binding Measurements

EGF was iodinated by the chloramine T method as described by Carpenter and Cohen [11]. In all experiments binding was at 0°C for 5 h, at which time binding had reached equilibrium. After 5 h 95% of ^{125}I-EGF was still on the cell surface as determined by washing with 0.2 N acetic acid/0.5 M NaCl. Cells were washed 2× with binding buffer (1 mM NaH_2PO_4, 85 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM glucose, 25 mM HEPES, 1 mg BSA/ml, 4°C) before incubation for 5 h at 0°C with binding buffer containing 3 ng/ml ^{125}I-EGF. Plates were then washed 2× with binding buffer and 3× with PBS (150 mM NaCl, 10 mM NaH_2PO_4, 4°C). The cells were then solubilized in 0.1 N NaOH and counted for ^{125}I. Nonspecific binding was determined in the presence of 500 ng unlabeled EGF/ml and was less than 10% of total binding. Counts were normalized to total protein determined by the method of Bradford [12].

RESULTS

BALB/c 3T3 cells infected with a Rous sarcoma virus expressing a temperature-sensitive mutant of $pp60^{v\text{-}src}$ (clone LA90) were used to examine the effect of activation of the oncogene protein on EGF and bradykinin binding. The in situ rate of activation of this $pp60^{v\text{-}src}$ kinase after switching the temperature of the cells from 40°C to 35°C is unusually rapid, with a half-time of less than 5 min [13], and the

increase in phosphorylation of cellular substrates is about threefold, as determined by immunoblotting with an antiphosphotyrosine antibody [13]. The activation of pp60^{v-src} is accompanied by a rapid, twofold decrease in the specific binding of ^{125}I-EGF (3 ng/ml), with a time course similar to that of pp60^{v-src} activation (Fig. 1); continued incubation at 35°C results in a slow, sustained decrease that is maintained indefinitely. Receptor binding at 3 ng EGF/ml to cells grown constitutively at permissive temperature is about one-fifth that of the control cell (40°C) value (Table II).

Uninfected parental BALB/c 3T3 cells and control NIH 3T3 cells both bind similar amounts of EGF at 35°C and 40°C (Fig. 1 and data not shown). To check that the decrease in binding to LA90 cells at 35°C was not a result of autocrine secretion of TGF-α or other growth factors, ^{125}I-EGF binding was measured using ts-src cells (grown at 40°C) that had been preincubated for one hour with conditioned, serum-free medium prepared from ts-src 3T3 cells grown at the permissive temperature for 48 h. The conditioned medium produced no significant decrease in ^{125}I-EGF binding compared to control values (data not shown).

By contrast to EGF binding, activation of pp60^{v-src} has no significant effect on either the number or affinity of bradykinin receptors (Table I), indicating that the effect on the EGF receptors is specific and is not mediated by, for instance, a rapid decrease in membrane surface area.

To examine whether the modulation of EGF binding by pp60^{v-src} is a consequence of altered receptor affinity or to a decreased receptor number, binding assays were performed on ts-src cells, grown at either 35°C or 40°C, over a range of EGF concentrations. At 40°C, binding to the ts-src cells is characterized by a single class of high-affinity sites (Kd ~0.17 nM) and cells grown at 35°C exhibit a decreased (~10-fold) affinity of the EGF receptor for its ligand with no significant decrease in the apparent number of surface receptors per cell (Fig. 2). A similar decrease in affinity,

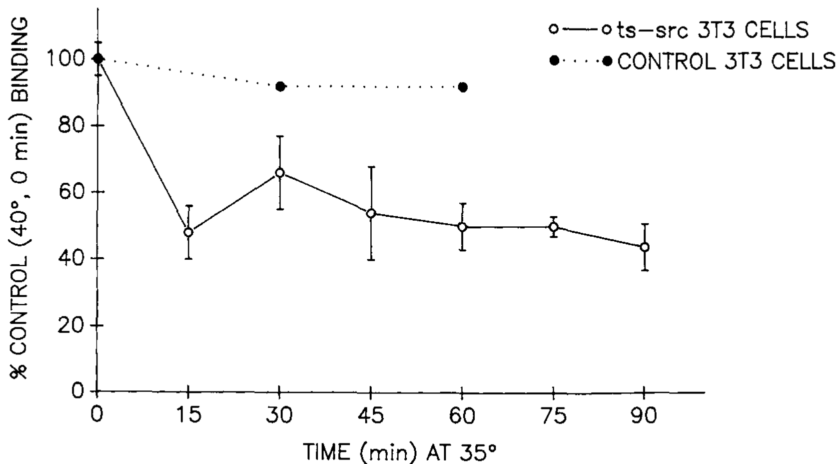

Fig. 1. Effect of temperature on specific binding of ^{125}I-EGF to its receptor in control (nontransformed) NIH 3T3 cells and LA90 ts-src 3T3 cells. Subconfluent cultures grown at 40°C were switched to 35°C by medium replacement and placed in a 35°C incubator for the indicated times. Specific binding of ^{125}I-EGF, at 3 ng/ml, was assayed as described in Materials and Methods after rapidly cooling the cells to 4°C. Nonspecific binding was determined in the presence of 500 ng unlabeled EGF/ml. Internalization of EGF receptors was responsible for less than 5% of the total specific ^{125}I-EGF binding. Counts were normalized to total protein determined by the method of Bradford [12].

TABLE I. Binding of [³H]-Bradykinin to LA90 3T3 Cells*

Treatment	$\bar{V}$ (fmol/mg cell protein)	Kd (nM)
40° → 40°	6.2	5
40° → 35° (5 hrs)	7.2	N.D.
40° → 35° (2 days)	6.4	5

*Specific binding of [³H]-bradykinin (88.7 Ci/mmol) was measured at 4°C for 2 h, as described by Roscher et al. [19], using 35-mm plates of LA90 cells, over a range of 1.25–30 nM. Values shown are means of duplicate determinations (range <15%). N.D., not determined.

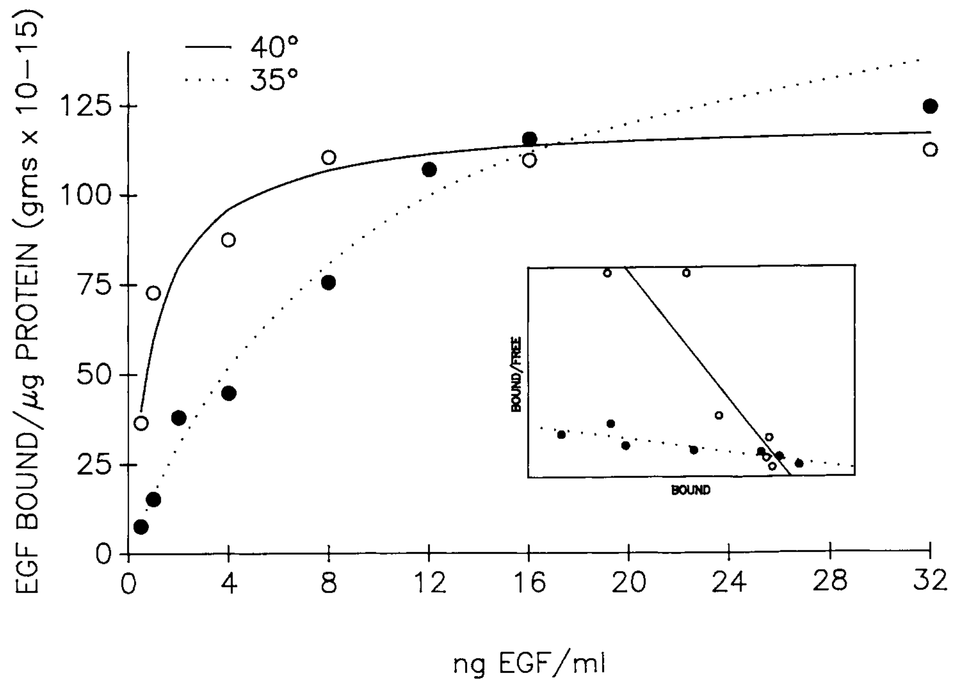

Fig. 2. Binding curves and Scatchard analysis of ^{125}I-EGF binding to cells grown at the restrictive (40°C) or permissive (35°C) temperature. Cells were grown for 2 days at 35°C or 40°C. EGF binding at 0°C was assayed as in Materials and Methods. Each point is the mean of six determinations and in no case was the standard deviation greater than 15% of the mean. Linear regression analysis of the Scatchard plot gave best-fit values for the dissociation constants (K_d) and binding site concentrations for cells grown 40°C and 35°C of 0.17 nM, 121 pg EGF bound/g cell protein (40°C, r = 0.907); and 1.6 nM, 180 pg EGF bound/g cell protein (35°C, r = 0.872), respectively.

in the absence of any change in total receptor concentration, was also observed for ts-src cells shifted from 40°C to 35°C for 15 min (data not shown). The absence of any down-regulation in the total cellular concentration of EGF receptor by $pp60^{v\text{-}src}$ has been confirmed by Western blotting [13].

The tumor-promoting phorbol esters, which are potent activators of PKC, also cause a rapid decrease in the binding of EGF to its receptor (Table II). Several experiments were therefore undertaken to determine whether $pp60^{v\text{-}src}$ down-regulates EGF binding through the activation of PKC. First, ts-src cells were grown at 40°C then

TABLE II. Effect of Phorbol Esters on pp60$^{v\text{-}src}$ Activated-down-Modulation of EGF Binding in ts-src 3T3 Cells*

	Pretreatment	Treatment	Proportion of control binding	% decrease
1. PKC depletion	40°C, 600 nM PMA 36 h	40°C	1.00 ± 0.14	
	40°C, 600 nM PMA 36 h	40°C + 200 nM PMA 15 min	1.10 ± 0.28	
	40°C, 600 nM PMA 36 h	35°	0.69 ± 0.18	
	40°C, 600 nM PMA 36 h	35°C + 200 nM PMA 15 min	0.70 ± 0.13	
2. src + PMA	40°C	40°C	1.00 ± 0.10	
	40°C	40°C + 200 nM PMA 15 min	0.13 ± 0.02	86
	35°C	35°	0.17 ± 0.03	
	35°C	35°C + 200 nM PMA 15 min	0.03 ± 0.01	82
3. PMA + src	40°C	40°C for 30 min	1.00 ± 0.04	
	40°C + 200 nM PMA 15 min	40°C for 30 min	0.35 ± 0.01	65
	40°C	35°C for 30 min	0.61 ± 0.07	
	40°C + 200 nM PMA 15 min	35°C for 30 min	0.22 ± 0.04	64

*1) Cells growing at each temperature were treated for 36 h with 600 nM PMA. After 36 h, the medium was changed to DMEM/0.1% CS ± 200 nM PMA at appropriate temperature. All treatments were for a total of 30 min. 2) Cells were grown in 35-mm dishes at pretreatment temperature, and PMA dissolved in DMEM was added to a final concentration of 200 nM. 3) All cells were grown in 35-mm plates. Two groups of cells were treated with PMA as in group 2. Temperature switch was effected by a medium change. In all cases, binding was assayed as in Materials and Methods. Values are means (n = 3) ± sample standard deviation. Control EGF binding values are 8.9, 9.7, and 9.4 pmol/mg protein, respectively.

pretreated for 36 h with 600 nM PMA to deplete them of protein kinase C. The chronic administration of phorbol esters dramatically increases the rate of degradation of PKC and can produce cells that are completely refractory, for several hours, to further stimulation by phorbol esters [14]. The PKC-depleted cells showed no significant decrease in ^{125}I-EGF binding in response to 200 nM PMA. Surprisingly, however, activation of the v-src kinase resulted in an approximately 30% drop in EGF binding within 30 min; and after 24 h at 35°C, the binding of EGF to PKC-depleted ts-*src* cells had fallen by about 70%, compared to control cells maintained in 600 nM PMA (Table II, and data not shown). Therefore, depletion of PKC does not prevent the decrease in EGF binding induced by active pp60$^{v\text{-}src}$. Moreover, the effects of PMA and pp60$^{v\text{-}src}$ activation on nondepleted ts-*src* cells are additive. As can be seen from Table II, 200 nM PMA induces a relative decrease in ^{125}I-EGF binding to ts-*src* cells grown at 40°C (~85%) similar to that in cells grown at 35°C. In the converse experiment, the decrease in EGF binding induced by activation of pp60$^{v\text{-}src}$ is also additional to that induced by 15-min pretreatment with 200 nM PMA. The v-src protein can, therefore, induce a decrease in the EGF binding affinity of 3T3 cells, the EGF receptors of which are already down-regulated by a saturating concentration of phorbol

ester. These experiments together provide strong evidence that the src protein does not act through PKC to down-regulate binding of EGF to its receptor.

To examine the reversibility of the down-regulation of EGF receptor affinity, the effect on ^{125}I-EGF binding was examined after reversing the temperature switch so as to inactivate pp60$^{v\text{-}src}$ kinase activity. Cells which were switched to 40°C after being grown for an extended time (48 h) at the permissive temperature required 36–72 h to recover the level of EGF binding exhibited by cells grown continuously at 40°C (Fig. 3A). This period is severalfold longer than the doubling time of these cells at 40°C. In Figure 3B, however, it can be seen that short-term activation of pp60$^{v\text{-}src}$, produced by switching cells from the restrictive to the permissive temperature for 15 min, is readily reversible when cells are switched back to the restrictive temperature, recovering control (40°C) levels of EGF binding in only 15 min. These results suggest that different mechanisms might control the short-term and long-term desensitization of the EGF receptor by pp60$^{v\text{-}src}$.

To investigate whether desensitization of EGF receptor affinity requires protein synthesis, ts-src 3T3 cells were treated with 20 μg cycloheximide/ml, to inhibit protein synthesis and ^{125}I-EGF binding was assayed as in previous experiments. Cells treated with cycloheximide exhibited the same rapid decrease in EGF binding on being switched from 40°C to 35°C as untreated cells, but surprisingly, binding recovered to normal levels in about 2 h in the absence of any further change in incubation temperature (Fig. 4).

DISCUSSION

It has been suggested that various oncogene products may induce expression of the transformed phenotype through the constitutive activation of transmembrane signalling systems, especially those utilizing the hydrolysis of the inositol lipids [15]. The product of the v-*src* oncogene, pp60$^{v\text{-}src}$, has been reported to increase PI turnover, to

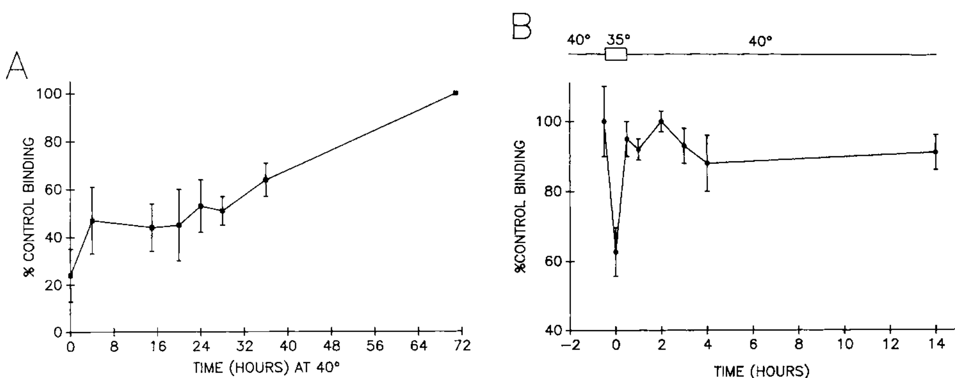

Fig. 3. Recovery of EGF binding to ts-src 3T3 cells after inactivation of pp60$^{v\text{-}src}$. **A:** Cells were grown at 35°C for 2 days. Plates were then moved to a 40°C incubator, without medium change, and left for indicated times prior to assaying EGF binding, as described in experimental procedures. **B:** Cells grown at 40°C were switched to 35°C by a medium change and after 30 min in the 35°C incubator were returned to the 40°C incubator without a medium change and left for the indicated amount of times, before determining EGF binding as described in Materials and Methods.

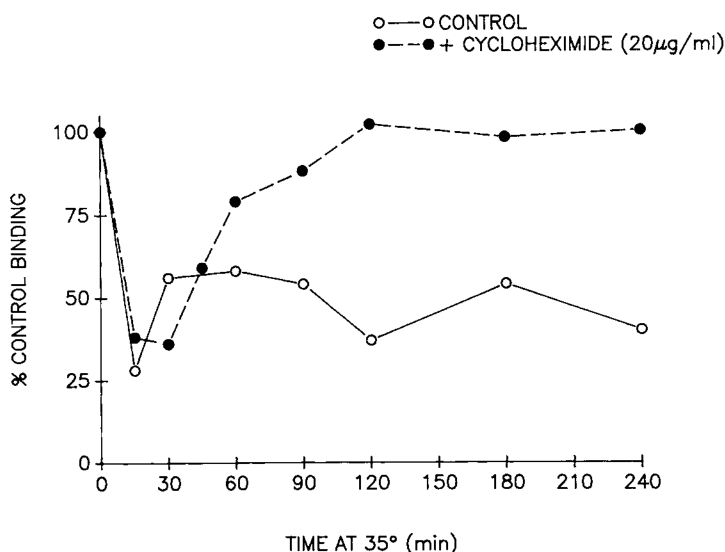

Fig. 4. Effect of cycloheximide on the pp60$^{v\text{-}src}$-induced down-regulation of EGF binding to ts-src cells. Cells grown at 40°C were switched to 35°C by changing medium to DMEM/0.1% CS ± 20 μg cycloheximide/ml at 35°C and placed in a 35°C incubator for the indicated time. Binding of ^{125}I-EGF was determined as in Materials and Methods. Points are means of duplicate determinations with a range of no greater than 9.5% about the mean.

increase steady-state levels of diacylglycerol, and to alter basal PKC activity, as measured by phosphorylation of an 80-kD substrate of PKC [4,5]. Because PKC is also known to phosphorylate the EGF receptor and thereby reduce the affinity of the receptor for EGF [7] we were interested in examining whether pp60$^{v\text{-}src}$ might induce a decrease in EGF binding via activation of PKC. We have now found that the activation of a temperature-sensitive pp60$^{v\text{-}src}$ tyrosyl kinase in BALB/c 3T3 fibroblasts very rapidly increases the tyrosyl phosphorylation of cell proteins [13] and causes a concurrent decrease in binding of ^{125}I-EGF to its receptor. This decrease is not an artifact of the temperature switch and is due neither to secretion of TGF-α, nor to a decrease in membrane surface area. No decrease was observed in the binding affinity or magnitude of bradykinin to its receptor in response to the activation of pp60$^{v\text{-}src}$. We conclude, therefore, that the decrease in binding of ^{125}I-EGF is a specific, direct effect on the EGF receptor of the activation of pp60$^{v\text{-}src}$ tyrosyl kinase activity.

Surprisingly, two distinct mechanisms of EGF receptor desensitization by pp60$^{v\text{-}src}$ can be distinguished based on the effects of cycloheximide and pp60$^{v\text{-}src}$ inactivation. One mechanism is rapid and transient, while the second is slow, persistent, and cycloheximide sensitive. Despite these differences, however, both mechanisms operate by decreasing EGF receptor affinity, and both appear to be independent of protein kinase C activity. The persistence of desensitization long after inactivation of the ts-pp60$^{v\text{-}src}$ indicates that the protein(s) responsible for this effect is very stable. Although the most obvious mechanism for long-term down-modulation of EGF receptor affinity would involve autocrine secretion of a factor such as TGF-α [16], or some other mitogenic peptide [17], we could find no evidence that conditioned medium from the transformed cells significantly affects ^{125}I-EGF binding. Moreover, because TGF-α, like

EGF, causes a rapid internalization and degradation of EGF receptors [18], the induction of TGF-α by pp60$^{v\text{-}src}$ would be expected to cause a decrease in the EGF receptor concentration, which is contrary to the results observed in our binding studies. We therefore suggest that the pp60$^{v\text{-}src}$-induced protein required for persistent EGF receptor desensitization is intracellular.

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