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Augmented Mitogenic Responsiveness to Epidermal Growth Factor in Murine Fibroblasts That Overexpress pp60$^{c\text{-}src}$

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C3H10T1/2 murine fibroblasts overexpressing chicken pp60$^{c\text{-}src}$ showed a two- to fivefold enhanced incorporation of [^{3}H]thymidine into DNA in response to epidermal growth factor (EGF) relative to that of the parent line. No difference in growth characteristics, number and affinity of EGF receptors, or hormone potency was attributable to c-*src* overexpression. These results suggest that pp60$^{c\text{-}src}$ may interact with the mitogenic signal transduction pathway of EGF in some event distal to hormone binding.

The proto-oncogene c-*src* encodes a 60-kilodalton phosphoprotein (pp60$^{c\text{-}src}$ [1]) that has been found in a wide variety of tissues (2, 6, 12) but whose role in cellular metabolism is unknown. Its location as a tyrosine kinase on the inner surface of the plasma membrane (3), a property shared with many activated growth factor receptors (10, 14), has prompted the speculation that it may be involved in mitogenic signal transduction. Although changes in the phosphorylation state or kinase activity of the c-*src* protein have been observed in cells treated with growth stimulatory agents such as phorbol esters or platelet-derived growth factor (5, 7, 16, 24), a direct role for pp60$^{c\text{-}src}$ in mitogenesis has not yet been demonstrated.

To determine whether the c-*src* protein is involved in the response of the cell to mitogenic stimulants such as epidermal growth factor (EGF), we generated clones of the murine fibroblast cell line C3H10T1/2 which overexpress pp60$^{c\text{-}src}$. This cell line was chosen for its high sensitivity to postconfluence inhibition of growth, low level of spontaneous transformation (17), and responsiveness to EGF and other growth factors (9). Cells were transfected by using the calcium phosphate precipitation technique (27) with plasmids containing either the genomic chicken c-*src* gene (pMc*src*) or the v-*src* gene of the Schmidt-Ruppin A strain of Rous sarcoma virus (pMv*src*) (both constructs were kindly provided by D. Shalloway [11]), along with pSV2*neo* (22), as described elsewhere (25). Neomycin-resistant colonies were screened for avian c-*src* or v-*src* expression by using monoclonal antibody EC10 (15). Five lines which expressed c-*src* protein 3- to 18-fold over the endogenous c-*src* level and one line which expressed v-*src* at a level 10-fold over the endogenous c-*src* level were established. Consistent with the inability of overexpressed pp60$^{c\text{-}src}$ to transform NIH 3T3 cells (11, 20), the C3H10T1/2 c-*src* overexpressors retained normal morphology, while the line transfected with v-*src*, IV5.3, was markedly altered (Fig. 1). The data summarized in Table 1 indicate that no significant differences between the C3H10T1/2 c-*src* overexpressors and the parent or *neo*-only (IN1.4) line with respect to saturation density, generation time, or growth in soft agar were observed, although c-*src* overexpressors that had been maintained in culture for 8 to 15 passages began to show limited anchorage-independent growth and a slight increase in both saturation density and DNA synthesis under serum deprivation. Therefore, all subsequent experiments were performed on cells that had undergone fewer than eight passages.

Mitogenic responsiveness to EGF. To measure the response to EGF, density-arrested monolayers were starved of serum for 24 h in Dulbecco modified Eagle medium supplemented with 0.1% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.), stimulated with 30 ng of EGF per ml (receptor grade; Collaborative Research, Waltham, Mass.) for 20 h, and labeled for 1 h with 1 μCi of [*methyl*-^{3}H]thymidine per ml (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.). The monolayers were washed, pelleted in cold STE buffer (150 mM NaCl, 50 mM Tris hydrochloride [pH 7.2], 1 mM EDTA), and suspended in 10% trichloroacetic acid. Precipitated material was collected, washed with 6% trichloroacetic acid on GF/A glass fiber filters (Whatman, Inc., Clifton, N.J.), digested in an NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, Ill.), and quantitated by scintillation counting. As indicated in Fig. 2 and Table 2, all of the c-*src* overexpressors showed a 20- to 50-fold increase in [^{3}H]thymidine incorporation after stimulation with EGF, reflecting a two- to fivefold enhancement of the 10-fold increase in hormone-induced DNA synthesis observed in the parent or *neo*-only line; no hormone effect was observed in the v-*src* overexpressor. Interestingly, no apparent correlation between the level of c-*src* overexpression and the degree of enhanced responsiveness could be made, suggesting that some other cellular component may have been limiting for the conveyance of the proliferative signal or, alternatively, that the avian c-*src* protein did not interact as effectively or as appropriately as endogenous pp60$^{c\text{-}src}$ with the murine constituents of the EGF mitogenic pathway. Similar results were also obtained when the numbers of nuclei which had incorporated [^{3}H]thymidine were quantitated in growth-arrested and EGF-stimulated cells. No difference in the percentages of labeled nuclei was observed between the c-*src* overexpressor, IC8.1, and the control lines, IN1.4 and C3H10T1/2, under nonstimulated conditions (Fig. 3), but in IC8.1 cells, stimulation with EGF resulted in a threefold enhancement in the percentage of cells in the population entering S-phase as compared with the percentages for IN1.4 and C3H10T1/2 cells (see reference 8 for the methods used). These results demonstrate that pp60$^{c\text{-}src}$ has the ability to sensitize quiescent cells to the proliferative signal of a defined growth factor, EGF.

Dose and kinetic response to EGF. Full-scale dose-response

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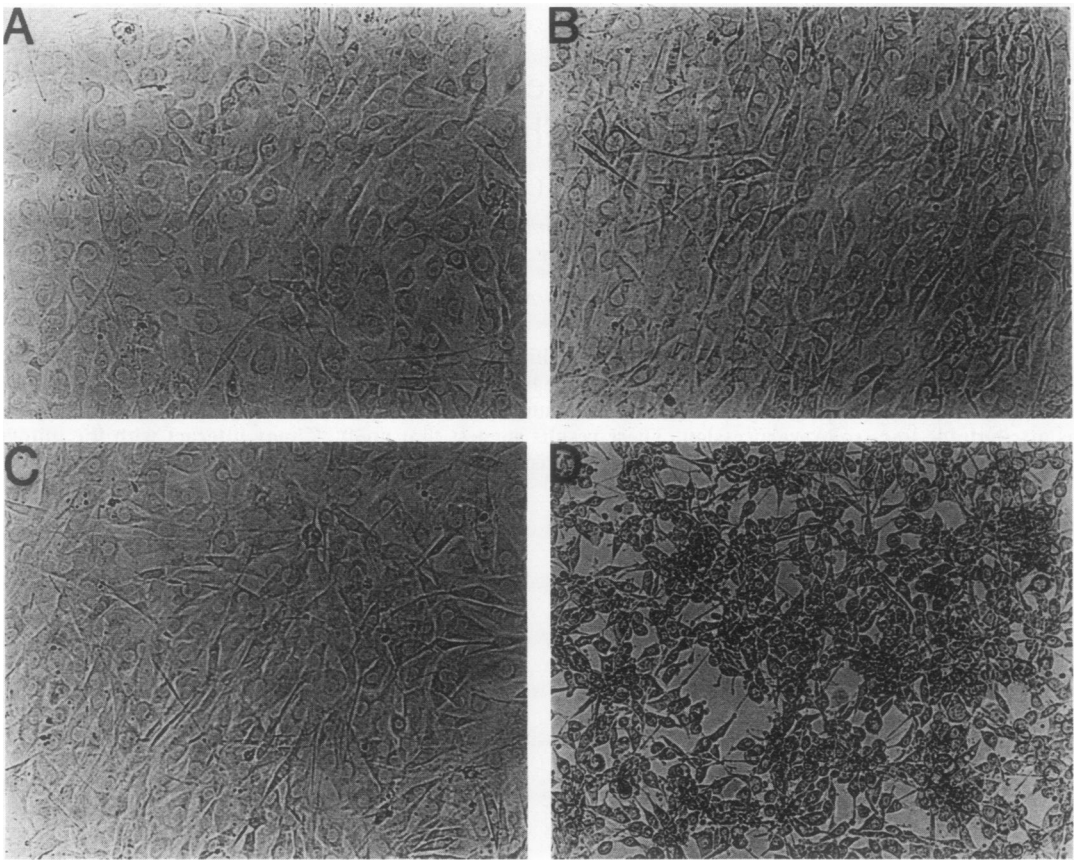

FIG. 1. Cellular morphology. (A) C3H10T1/2 cells; (B) IC8.1, c-*src* transfectant; (C) IN1.4, *neo*-only transfectant; (D) IV5.3, v-*src* transfectant.

analyses and time course studies were performed with IC8.1 and C3H10T1/2 cells. While c-*src* overexpression enhanced the magnitude of the mitogenic response to EGF, no significant effect on the hormone potency or time course of the response was observed. Both cell lines exhibited a half-maximal response at an EGF concentration of 3×10^{-10} M (data not shown) and initiated DNA synthesis 14 to 16 h after EGF stimulation (data not shown).

EGF binding characteristics. ^{125}I-EGF (165 mCi/mg; New England Nuclear) competitive binding studies were performed as previously described (23) with IC8.1, VC37.2, C3H10T1/2, and IN1.4 cells. While a slight alteration in binding affinity could not be resolved by this assay (Fig. 4), no appreciable effect on EGF binding affinity or receptor number could be attributed to c-*src* overexpression. Thus, the mechanism by which the enhanced mitogenic response to EGF is mediated appears to occur at some point after hormone binding.

Additional evidence generated in our laboratory indicated that the c-*src* protein may indeed be involved in the EGF mechanism of action. First, we observed transient, cyclical changes in the kinase activity of pp60$^{c\text{-}src}$ immunoprecipitated from Swiss 3T3, normal rat kidney, and chick embryo cells within 30 min of treatment with EGF. In multiple

TABLE 1. Biological characteristics of transfected C3H10T1/2 cell lines

Cell line	Transfected DNA	Level of *src* expression[a]	Generation time (h)	Saturation density[b]	Anchorage-independent growth (% colony formation)
C3H10T1/2		1	13.8	3.1×10^5	0
IN1.4	pSV2*neo*	1	ND[c]	2.6×10^5	ND
IC8.1	pSV2*neo* + pMc*src*	18	15.3	2.1×10^5	0
VC24.3	pSV2*neo* + pMc*src*	12	14.7	1.6×10^5	0
VC25.5	pSV2*neo* + pMc*src*	16	16.5	2.5×10^5	0
VC35.4	pSV2*neo* + pMc*src*	6	14.7	2.5×10^5	0
VC37.2	pSV2*neo* + pMc*src*	3	14.0	3.3×10^5	0
IV5.3	pSV2*neo* + pMv*src*	10	23.8	1.9×10^6	15

[a] Expressed as a fold increase over the endogenous level of rodent pp60$^{c\text{-}src}$ (0.001% of total cell protein). Levels of *src* expression were determined by [^{35}S]methionine metabolic labeling as described elsewhere (13).
[b] Expressed as the number of cells per 35-mm culture dish.
[c] ND, Not determined.

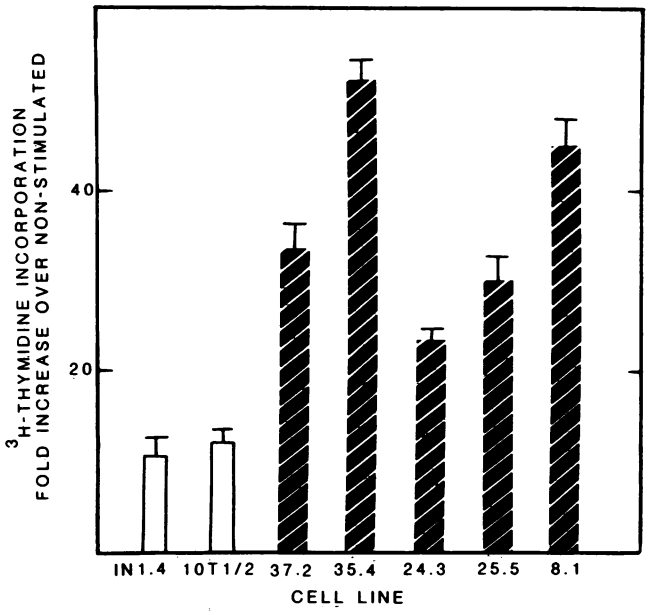

FIG. 2. [^{3}H]thymidine incorporation into total DNA in response to EGF. The data represent the mean increases (n = 3) ± the standard errors in [^{3}H]thymidine incorporation into DNA after stimulation relative to the nonstimulated level for each cell line from a representative experiment. C3H10T1/2 is the parent line; IN1.4 is the *neo*-only transfectant; 24.3, 25.5, 35.4, 37.2, 8.1 refer to c-*src* transfectants VC24.3, VC25.5, VC35.4, VC37.2, and IC8.1, respectively.

TABLE 2. EGF responsiveness of transfected C3H10T/2 cell lines

Cell line (n)	[^{3}H]thymidine incorporation (cpm/10^5 cells ± SE)	
	Basal	EGF stimulated
C3H10T/2 (5)	442 ± 110	5,456 ± 1,168[a]
IC8.1 (5)	479 ± 138	19,240 ± 4,700[a]
IV5.3 (2)	10,901 ± 1,304	10,313 ± 1,152

[a] Mean of five experiments, each done in triplicate. Paired t statistics determined for the response of C3H10T1/2 and IC8.1 cells indicate a significant difference ($P < 0.01$).

experiments, a twofold increase in the autokinase activity of the c-*src* protein was apparent within 1 min after hormone addition, followed by a return to nonstimulated levels by 3 min. A second cycle of activation with a maximum 10-fold increase in autokinase activity occurred 6 to 10 min after stimulation, and by 30 min poststimulation, activity had returned to basal levels (unpublished data). This effect, however, has been difficult to reproduce consistently, a difficulty most likely due to the time and handling required to prepare the lysates before the actual kinase reaction. A second line of evidence is that a 6- to 30-fold constitutive decrease in the specific activity of pp60$^{c\text{-}src}$ has been found in quail cells infected with avian erythroblastosis virus (13). The fact that this striking alteration in activity of the c-*src* protein occurred after transformation with a virus which contains an oncogene, v-*erbB*, whose product is thought to function as a constitutively activated EGF receptor (4), again suggests that pp60$^{c\text{-}src}$ may interact with the EGF signal transduction pathway.

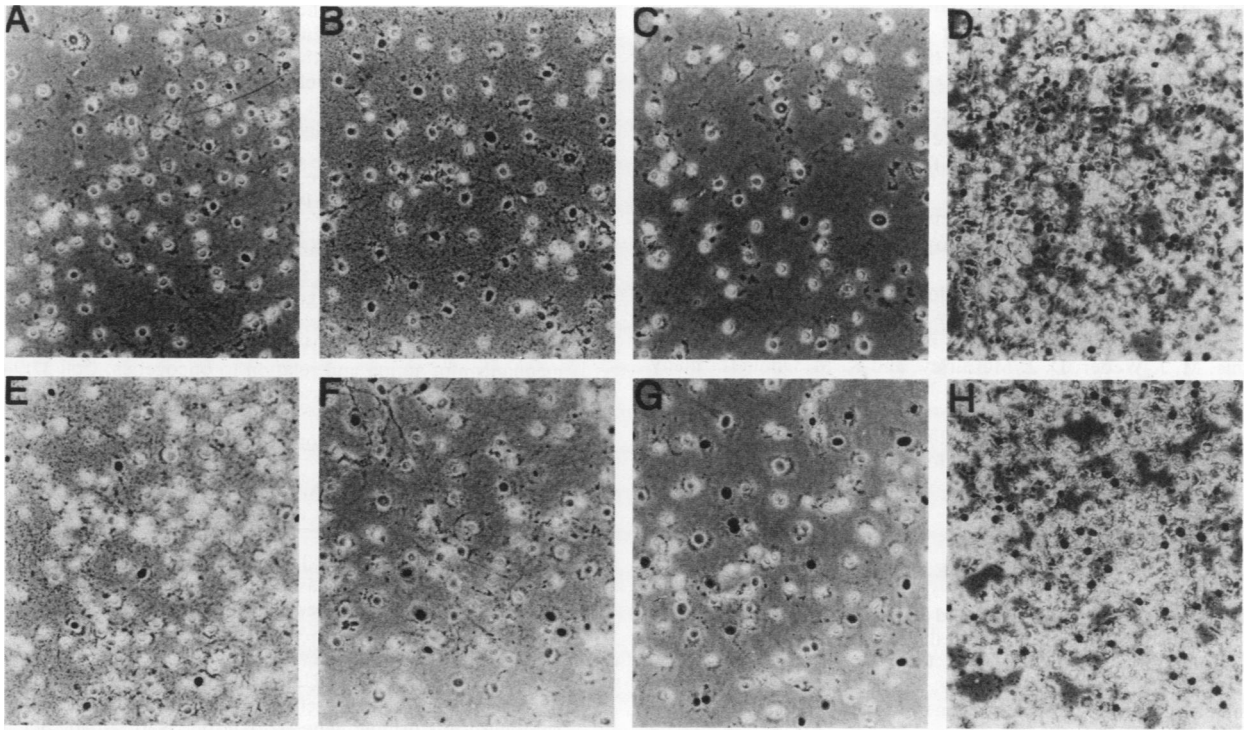

FIG. 3. [^{3}H]thymidine incorporation into nuclei. The percentages of labeled nuclei were determined by scoring 500 cells per plate and averaging the results from duplicate plates. (A) C3H10T1/2 parent line, nonstimulated (0.9%); (E) C3H10T1/2 parent line, EGF stimulated (7.9%); (B) IN1.4, nonstimulated (1.3%); (F) IN1.4, EGF stimulated (9.3%); (C) IC8.1, nonstimulated (1.2%); (G) IC8.1, EGF stimulated (21.0%); (D) IV5.3, nonstimulated (not determined); (H) IV5.3, EGF stimulated (not determined).

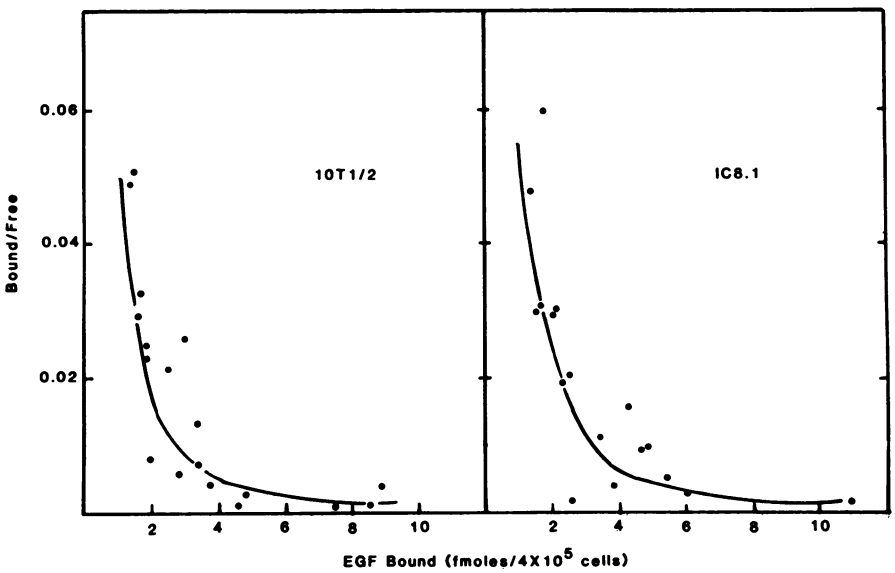

FIG. 4. ^{125}I-EGF bound to C3H10T1/2 and c-*src* overexpressor IC8.1. Equilibrium binding of ^{125}I-EGF to intact cell monolayers was determined in the presence of increasing concentrations of unlabeled EGF. Data were corrected for nonspecific binding, determined in the presence of 3×10^{-7} M unlabeled ligand. Results depict representative Scatchard analyses (19) of ^{125}I-EGF binding to C3H10T1/2 and IC8.1 cells; similar results were obtained for c-*src* overexpressor VC37.2 and the *neo*-only transfected line IN1.4. All cells exhibited both high- and low-affinity receptors, with a K_d of 30 pmol and 5,000 sites per cell for the high-affinity component and a K_d of 30 nmol and 60,000 sites per cell for the low-affinity component.

Overexpression of proto-oncogenes other than c-*src* can also affect the normal response of the cell to growth factors. Overexpression of c-*myc* in C3H10T1/2 cells has been shown to increase the capacity for anchorage-independent growth in these cells in the presence of a variety of competence and progression factors (21). An enhanced mitogenic response to EGF has also been observed in NIH 3T3 c-N-*ras* overexpressor cells under conditions of possible serum-induced inositol phospholipid turnover (26). Several studies have demonstrated a synergistic mitogenic effect between growth-stimulating agents which promote phosphatidylinositol turnover and EGF (see reference 18 for a review). It is possible that overexpression of pp60$^{c\text{-}src}$ alters the levels of phosphatidylinositol turnover or other second messengers in the cell, thus potentiating the response to EGF.

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