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Inhibition of Epidermal Growth Factor Receptor Biosynthesis Caused by the *src* Oncogene Product, pp60$^{v\text{-}src}$

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We have previously shown that an intracellular mechanism down regulates epidermal growth factor (EGF) receptor levels in rodent fibroblasts transformed by the *src* oncogene (W. J. Wasilenko, L. K. Shawver, and M. J. Weber, J. Cell. Physiol. 131:450–457, 1987). We now report that this down regulation is due to an inhibition of EGF receptor biosynthesis. With Rat-1 (R1) cells infected with a temperature-sensitive *src* mutant, we found that ^{125}I-labeled EGF binding to cells began to decrease soon after the activation of pp60$^{v\text{-}src}$ by shift down to the permissive temperature for transformation. This effect of *src* on EGF receptors was reversible. Pulse-chase studies with [^{35}S]methionine-labeled cells revealed that the tyrosine protein kinase activity of pp60$^{v\text{-}src}$ had little if any effect on EGF receptor degradation rate. By contrast, the expression of pp60$^{v\text{-}src}$ caused a large reduction in the apparent rate of EGF receptor biosynthesis. Northern (RNA) blot analysis demonstrated that pp60$^{v\text{-}src}$ also caused marked reductions in the steady-state level of EGF receptor mRNA. These data indicate that one way the expression of the *src* oncogene can affect the machinery of growth control is by affecting the expression of specific genes for growth factor receptors.

Oncogenes transform cells by virtue of their ability to mimic or interact with components of the machinery of normal growth regulation (10, 11, 22). Among the most thoroughly studied oncogenic agents is the *src* oncogene of Rous sarcoma virus, which encodes pp60$^{v\text{-}src}$, a 60-kilodalton tyrosine-specific protein kinase that associates with the inner surface of cellular membranes (8, 21, 34, 35). Both the tyrosine protein kinase activity and the membrane association of this protein are required for transformation, but little else is known about the mechanism(s) by which pp60$^{v\text{-}src}$ disrupts normal cellular proliferation. In particular, it is unclear which step(s) in the control of normal growth and metabolism is directly altered by pp60$^{v\text{-}src}$. As a starting point for the investigation of this problem, we have examined the effects of pp60$^{v\text{-}src}$ on the epidermal growth factor (EGF) receptor, since this well-characterized receptor is also a tyrosyl protein kinase (42) and an important determinant of growth regulation in a variety of normal and malignant cells (5, 6, 23, 27). Moreover, published reports from S. J. Parsons and co-workers indicate that overexpression of c-*src* potentiates the mitogenic effects of EGF (28). These findings raise the possibility of interactions between *src* and the EGF receptor in the control of cellular growth and metabolism.

In earlier studies, we reported that EGF receptor levels were markedly down modulated in rodent cells transformed by pp60$^{v\text{-}src}$ (43). Although the basis for this down modulation was unclear, it appeared to be dependent on the tyrosine kinase activity of pp60$^{v\text{-}src}$. In addition, we found little (if any) role for secreted transforming growth factors (39) in this phenomenon, leading us to suggest that the mechanism for receptor loss was based largely on intracellular events mediated by pp60$^{v\text{-}src}$.

In this study, we sought to better define the molecular basis for decreased EGF receptor expression in *src*-transformed cells. Our approach was to examine several parameters of EGF receptor metabolism, including the turnover and biosynthesis of the EGF receptor, in Rat-1 (R1) fibroblasts infected with the temperature-sensitive *src* mutant, LA29 (37). These cells are phenotypically normal at culture temperatures of 40°C but are fully transformed at 35°C with respect to morphology, glucose transport, and growth properties (44; our unpublished data).

To determine the kinetics of *src*-mediated modulations of EGF receptors in R1/LA29 cells, temperature-shift studies measuring ^{125}I-labeled EGF binding to these cells were performed. Significantly less EGF binding occurred in R1/LA29 cells cultured at 35°C compared with those cultured at 40°C (Fig. 1). Shifting the cells from the restrictive temperature to the permissive temperature for transformation led to a loss of EGF binding over time, such that by 18 h, this down modulation was nearly complete. Conversely, the EGF-binding capacity of these cells increased steadily after shift up from 35 to 40°C. These finding indicate that the effects of *src* on EGF receptors in rat cells are reversible and dependent on the activity of pp60$^{v\text{-}src}$. Repeated attempts to further analyze these ligand-binding changes by Scatchard analysis (33) were unsuccessful because of the low endogenous level of receptors in these cells.

The effects of *src* expression on growth factor binding appear not be a general effect on all growth factor receptors, as binding of platelet-derived growth factor and insulin is not greatly altered in *src*-transformed cells (our unpublished data).

The apparent reduction in the steady-state level of cellular EGF receptors demonstrated above could have come about by an increase in the turnover of the protein, a reduced level of its biosynthesis, or both. Therefore, we determined the effects of *src* on both of these parameters of EGF receptor metabolism. To examine receptor turnover, pulse-chase experiments were performed in which R1/LA29 cells were grown at 40°C and then labeled with [^{35}S]methionine for 12 h at this temperature. After the cells were labeled, the cultures

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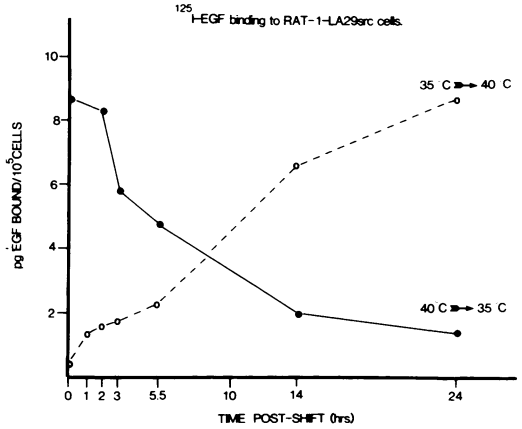

FIG. 1. Time course of changes in ^{125}I-EGF binding to R1/LA29 cells. R1/LA29 cells were constructed by infection of R1 cells with Rous sarcoma virus carrying a temperature-conditional *src* gene (ts LA29), in a subgroup D pseudotype (37). The permissive temperature for transformation of these cells is 35 to 35.5°C, and the restrictive temperature is 39.5°C (referred to as 40°C in this report). For the EGF binding assay, the cells were grown to near confluency as previously described (43) at either the restrictive temperature (40°C) or the permissive temperature (35°C) for pp60$^{v\text{-}src}$ activity. At various times before binding assays were performed (1.5 to 24 h), some cell cultures were shifted to the alternate temperature. For ^{125}I-labeled EGF-binding measurements, cells were incubated with ^{125}I-labeled EGF (2 ng/ml) (Dupont, NEN Research Products) in binding medium (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-buffered [20 mM] Dulbecco medium [pH 7.4], 0.1% bovine serum albumin) for 1.5 h at 22°C. After the cells were incubated, binding medium was removed and the cells were rinsed 3 times with cold phosphate-buffered saline–0.1% bovine serum albumin and lysed in 0.5N NaOH (1.5 h at room temperature). Cell-associated radioactivity in lysates was then determined. The data, corrected for nonspecific binding in the presence of a 100-fold excess of unlabeled EGF, represent the means of duplicate determinations, which generally varied by less than 10%.

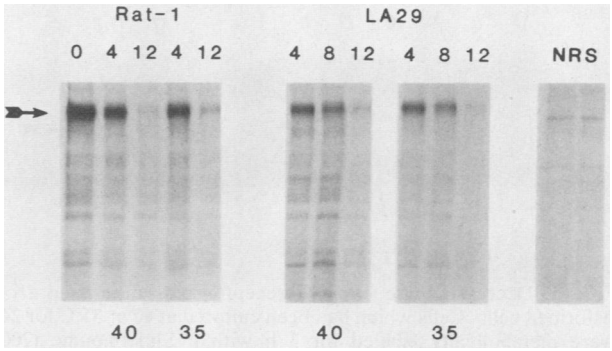

FIG. 2. Turnover of EGF receptors in R1/LA29 cells cultured at the permissive and nonpermissive temperatures for *src* transformation. Cell cultures of R1/LA29 cells were grown at 40°C for 24 h and then labeled for 12 h at this temperature with [^{35}S]methionine (250 μCi/ml) in Dulbecco medium containing 5% of the regular level of methionine and 10% dialyzed fetal bovine serum. After the cultures were labeled and rinsed, the cell cultures were then refed with fresh culture medium containing nonradioactive methionine (time zero). Half of the cell cultures were shifted to 35°C for additional incubation, while the remaining cultures were kept at 40°C. At varying times during the chase incubation (3 to 12 h), some of the cell cultures were lysed in ice-cold RIPA (0.1% sodium dodecyl sulfate, 0.15 M NaCl, 50 mM Tris hydrochloride [pH 7.2], 1% Triton X-100, 1% deoxycholic acid) supplemented with 200 KIU of aprotinin per ml. Lysed cells were stored at −20°C. Immunoprecipitations were performed on thawed cell lysates, essentially as described previously (43), with an antiserum raised against rat liver EGF receptors (generously provided by H. S. Earp [13, 14]). The immunoprecipitated material was fractionated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis and analyzed after autoradiography. Included as a control for possible temperature artifacts were cultures of normal R1 cells labeled and chased identically. NRS, Normal rabbit serum control. The EGF receptor is marked with an arrow.

were changed to fresh medium containing unlabeled methionine; half were shifted to 35°C, while the other half were kept at 40°C. At various times during the chase, both groups were lysed and the radioactivity remaining in the EGF receptor was assessed by immunoprecipitation followed by gel electrophoresis and autoradiography.

The rate of decay of radiolabeled receptor in R1/LA29 cells was similar during both incubations (at 35 and 40°C) with an estimated half-life of approximately 7 h. This is very close to the half-life determined by analysis of ^{125}I-labeled EGF binding (Fig. 2). Since the turnover of receptor protein in normal R1 cells was not affected by temperature (Fig. 2), it appears from the data that an increased turnover of receptor protein does not occur after pp60$^{v\text{-}src}$ activation. This result was somewhat unexpected, as Glenney et al. (19) have reported evidence which indicates a requirement for tyrosine phosphorylation in the internalization of the EGF receptor. Since we have found that the preponderance of proteins which become tyrosine phosphorylated in response to EGF also becomes phosphorylated in response to pp60$^{v\text{-}src}$ (our unpublished data), we anticipated that receptor turnover would be affected in cells expressing pp60$^{v\text{-}src}$. Perhaps specific differences between pp60$^{v\text{-}src}$ and the EGF receptor in the sites or proteins which become tyrosine phosphorylated account for this result, or perhaps the tyrosine phosphorylation is necessary but not sufficient for receptor internalization.

The fact that pp60$^{v\text{-}src}$ does not alter turnover of the EGF receptor is consistent with our earlier suggestion that down modulation of receptors in these cells does not involve production of transforming growth factor-alpha (43).

To address the effects of pp60$^{v\text{-}src}$ on EGF receptor biosynthesis, R1/LA29 cells were grown for at least 24 h at either 40 or 35°C and were then pulse-labeled with [^{35}S] methionine for 3 h. Control experiments demonstrated approximately linear synthesis of precipitable labeled receptor during this time. After the cells were labeled, they were lysed and the level of de novo synthesized receptor was analyzed by immunoprecipitation. To account for possible differences in methionine utilization or overall protein synthetic rates, equivalent levels of trichloroacetic acid-precipitable radioactivity were used for the immunoprecipitations. Similar analyses were also performed on R1 cells overexpressing the wild-type v-*src* oncogene.

The results of this analysis are shown in Fig. 3. It can clearly be seen that a markedly lower level of radiolabeled EGF receptor is made in R1/LA29 cells grown at 35°C (Fig. 3, lane b) and in R1 cells transfected with v-*src* (Fig. 3, lane e) as compared with that made in normal R1 cells (Fig. 3, lane c or d) or R1/LA29 cells grown at 40°C (Fig. 3, lane a). It is worthwhile to note that overexpression of c-*src* in rodent fibroblasts did not cause a marked inhibition of receptor biosynthesis or a decrease in ^{125}I-labeled EGF binding (data not shown). Controls for temperature artifact indicate that the level of EGF receptor synthesis is similar in R1 cells grown and labeled at 40 or 35°C. In addition,

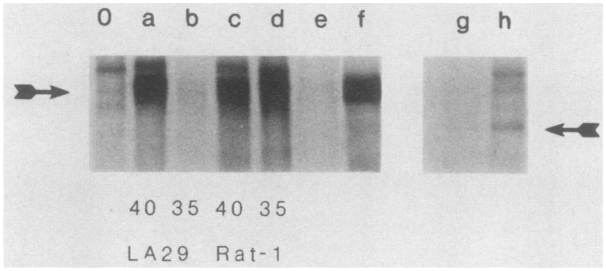

FIG. 3. Decreased rate of EGF receptor biosynthesis in *src*-transformed cells. Cells which had been cultured at 40 or 35°C for 24 h were metabolically labeled for 3 h with [^{35}S]methionine (200 μCi/ml). The cells were then lysed, and the level of newly synthesized receptor in the cell lysates was analyzed by immunoprecipitation and autoradiography. An equal amount of trichloroacetic acid-precipitable radioactivity from all of the cell types was used in the immunoprecipitations. Lanes: 0, normal serum control; a and b, R1/LA29 at 40 and 35°C; c and d, R1 at 40 and 35°C; e, R1/v-*src*; f, A431 cells; g and h, R1/LA29 and R1 at 35°C treated with tunicamycin. Similar results were observed even after shorter pulse-labeling (1.5 h) (data not shown). The EGF receptor is marked with an arrow.

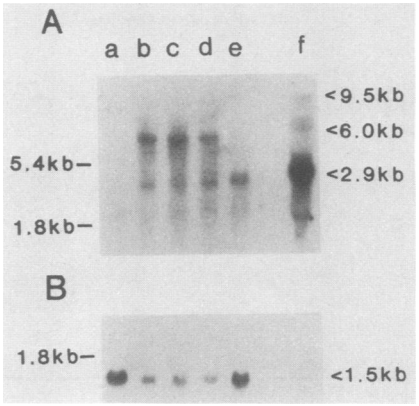

FIG. 4. Northern blot analysis of EGF receptor mRNA levels in normal and *src*-transformed R1 cells. Total cytoplasmic RNA was extracted from cells cultured for 48 h at each temperature by the method described by Maniatis et al. (29). Poly(A)$^+$ RNA was subsequently selected by passage of cytoplasmic RNA through oligo(dT) columns (type III; Collaborative Research, Inc.). The selected poly(A)$^+$ RNA (15 μg) was electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon (Nytran) membrane filters. The filters were prehybridized for 18 h at 42°C in 50% formaldehyde, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), 5× Denhardt medium, 1% sodium dodecyl sulfate, and 200 μg of denatured salmon sperm DNA per ml. Hybridizations were carried out under the same conditions and in the same solution containing, in addition, probes labeled with ^{32}P by nick translation. Filters were first washed twice (10 min each) at 56°C in 2× SSC–0.1% sodium dodecyl sulfate. Finally, they were washed two times (5 min each) at room temperature in 0.2× SSC–0.1% sodium dodecyl sulfate. Filters were dabbed dry and exposed to Kodak X-RP film with an intensifying screen at −70°C. The probes used were a 2.3-kb *Eco*RI fragment isolated from prEGF-R4.2 (A), containing rat EGF receptor cDNA (generously provided by H. S. Earp [14]) and a 1.1-kb fragment isolated from pGAD28 (B), containing the chicken GAPDH cDNA (12). Poly(A)$^+$ selected RNAs (15 μg) from R1 (v-*src*) cells (lane a), R1 cells grown at 40°C (lane b) or 35°C (lane c), R1/LA29 cells grown at 40°C (lane d) or 35°C (lane e). Lane f, RNA from human A431 cells. The relevant mRNA species and the positions of the internal markers (rRNAs) are marked.

R1/LA29 cells (35°C) treated with tunicamycin, an N-linked glycosylation inhibitor, made less of the 130-kilodalton aporeceptor species (Fig. 3, lane g) than did R1 cells treated similarly (Fig. 3, lane h), indicating that decreased synthesis of the receptor was not due to the effects of pp60$^{v\text{-}src}$ on receptor processing. Taken together, these findings indicate that the activity of the pp60$^{v\text{-}src}$ kinase causes a major reduction in the rate of EGF receptor biosynthesis in R1 cells.

Since *src* was found to reduce EGF receptor biosynthesis, it was important to determine if this reduction was related to a change in the level and/or species of EGF receptor mRNA present in *src*-transformed cells. To examine this, Northern (RNA) blot hybridizations (Fig. 4) were conducted with poly(A)$^+$-selected mRNA from R1/v-*src* cells (Fig. 4, lane a), R1 cells at 40 and 35°C (Fig. 4, lanes b and c), and R1/LA29 cells at 40 and 35°C (Fig. 4, lanes d and e). Cytoplasmic RNA from A431 cells was used as a qualitative size marker for the various messages (Fig. 4, lane f). The probe used to analyze the effect of *src* on steady-state levels of EGF receptor mRNA expression was a partial cDNA for rat EGF receptors (13, 14). With this probe, we detected hybridizing mRNA forms of approximately 6 and 2.9 kilobases (kb) in normal R1 cells grown at either 40 or 35°C. We were also able to see a faint band corresponding to a mRNA species of approximately 9.5 kb. Similarly sized EGF receptor mRNAs have been found in other types of rat cells with rat and human receptor probes (13, 14, 26). In poly(A)$^+$ RNA from these cells probed with human cDNA, we detected the 6 and 9.5-kb species but not the 2.9-kb RNA (data not shown). The origin of the 2.9-kb RNA is unclear; it could be RNA related to the EGF receptor, which is detected by the homologous probe but not by the human probe, or it could be related to the truncated RNA reported by others in A431 cells (31). In any event, this species is too small to encode the full-length EGF receptor.

The Northern blots were stripped of the EGF receptor probe and rehybridized with labeled DNA encoding the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (12) (Fig. 4B). This enzyme is often considered an invariant housekeeping enzyme (although we have noted its increased levels in the RNA from the transformed cells). The autoradiographs were subjected to densitometric analysis and the GAPDH mRNA level in R1 cells grown at 40°C was used as the baseline to normalize other changes in gene expression (Table 1).

The level of EGF receptor 6-kb mRNA species was reduced significantly (10- to 20-fold) in both transformed cell lines, R1/v-*src* and R1/LA29, grown at the permissive tem-

TABLE 1. Relative amounts of RNA species

Cell lines	Temperature (°C)	Abundance of the following mRNA[a]		
		GAPDH[b]	EGFR (6.0 kb)[b]	EGFR (6.0 kb)[c]
R1	40	1.00	2.19	2.19
R1	35	0.94	2.40	2.55
R1/LA29	40	0.83	4.91	2.30
R1/LA29	35	2.54	0.21	0.08
R1/v-*src*	35	3.13	0.17	0.05

[a] The autoradiograms were subjected to densitometric analysis and the abundance of the EGF receptor (EGFR) mRNA species is given.
[b] In arbitrary densitometric units.
[c] As normalized for variations in the GAPDH mRNA levels.

perature (35°C). The level of the 9.5-kb species was also reduced by transformation, but the signal from this mRNA form was too low to quantitate. Qualitatively identical results were obtained with both full-length and partial human cDNA probes (data not shown). These results suggest that a decrease in EGF receptor gene expression underlies the *src*-induced loss of EGF receptors from cultured cells.

Altered expression of key cellular regulatory or metabolic genes is not uncommon in *src*-transformed cells. Studies with fibroblasts in culture have shown that *src* stimulates expression of the genes for glucose transporters (16, 44), cathepsin (17), transin (30), GRP78 (36), and a mitogenic platelet protein (3, 38). In the present study, we also found that *src* transformation results in a modestly increased expression of the mRNA for GAPDH. This latter change may be related to the changes in glycolysis which are characteristic of the transformed cells.

In certain *src*-transformed fibroblasts, down-regulated gene expression has also been reported for fibronectin (1, 2, 15, 40, 41) and type 1 collagen (1, 2, 32), which are extracellular matrix components, the cytoskeletal protein tropomyosin (24), and a quiescence-specific gene (4). Surprisingly, it appears from our findings that the EGF receptor is also a member of the down-regulated class of genes, even though the receptor shares no known structural or functional properties with these other types of *src*-inhibited gene products. Conversely, expression of genes encoding receptors for platelet-derived growth factor and insulin are unlikely to be affected by *src*, as binding of these ligands was not greatly altered in *src*-transformed cells (data not shown). In any event, studies with chondroblasts and chick embryo fibroblasts suggest that the pattern of genes affected by *src* can vary depending on the differentiation program or lineage of the cell type (1, 2, 15). Thus, if EGF receptors and other *src*-inhibited proteins are markers of a differentiated fibroblast, their coordinated loss from *src*-transformed cells may reflect a dedifferentiation required for continuous cell division.

The regulatory significance of the *src*-induced change in receptor biosynthesis is uncertain. We have found that the EGF receptor does not inhibit *src* transformation, since we have observed that overexpression of a human EGF receptor under the control of a viral long terminal repeat does not interfere with *src*-mediated transformation in R1/LA29 cells (data not shown). (Interestingly, and in contrast to our results, the expression of the EGF receptor may be required for transformation by the *abl* oncogene [18, 43].) Our findings do, however, add to a growing body of evidence for some link between $pp60^{v\text{-}src}$ and the EGF mitogenic pathway. This evidence includes reports of EGF receptor desensitization and decreased EGF binding in *src*-transformed cells (9, 20) and the recent discovery that the overexpression of $pp60^{c\text{-}src}$ sensitizes certain murine fibroblasts to the mitogenic effects of EGF (28). Thus, our discovery of EGF receptor down modulation in *src*-transformed cells may be symptomatic of regulatory communication between the EGF receptor and $pp60^{src}$ signalling systems.

The mechanism by which $pp60^{v\text{-}src}$ inhibits EGF receptor gene expression is currently unknown. Apparently, the initiation of this change in gene activity is rapid but perhaps not immediate, since a substantial net loss of receptor from the cell surface is detectable within 3 h after shift down of R1/LA29 cells to the permissive temperature. In chick embryo fibroblasts, reductions in fibronectin mRNA occur within as little as 5 h after the activation of $pp60^{v\text{-}src}$ (41). Because of these kinetics for mRNA changes, the fact that temperature-dependent changes in cellular tyrosine phosphorylation are detectable within 1 h (our unpublished data) and the fact that $pp60^{v\text{-}src}$ is localized at the cell membrane, we suspect that this oncogene decreases EGF receptor mRNA levels indirectly. This decrease might come about by changes in the level or properties of second messengers, which could then act to decrease receptor gene expression by affecting the initiation of transcription, attenuation of transcription, or mRNA stability.

Reduced initiation of transcription has been implicated in the loss of at least two other gene products, fibronectin and collagen, from *src*-transformed cells (32, 40). However, expression plasmids containing the chloramphenicol acetyltransferase gene under the control of the EGF receptor promoter region failed to display any modulation in response to *src* transformation (our unpublished data). This suggests that *src* alters EGF receptor gene expression at some step after the initiation of transcription, as does EGF itself (25). However, comparative studies involving nuclear run-on assays and analysis of mRNA stability are required to determine the basis for the down regulation of EGF receptor gene expression reported here.

Previous studies on the regulation of EGF receptor gene expression have revealed that activation of EGF receptors by ligand binding causes an increase in the cellular level of EGF receptor mRNA (7, 13, 14, 26). This increase presumably occurs via tyrosine phosphorylation induced by the tyrosine kinase activity of the receptor. Our finding that $pp60^{v\text{-}src}$ causes the opposite effect on the level of steady-state receptor mRNA points toward a complex role for tyrosine phosphorylations in the regulation of EGF receptor mRNA levels; although both $pp60^{v\text{-}src}$ and overexpressed EGF receptor can transform cells, they must send signals which have at least some contrasting effects on the regulation of gene expression. Further studies on the control of EGF receptor mRNA levels in *src*-transformed cells should provide interesting information on the mechanism(s) by which tyrosine kinases can alter gene expression.

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