Similarities and Differences between the Effects of Epidermal Growth Factor and Rous Sarcoma Virus

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ABSTRACT We have derived a line of A431 human tumor cells infected with Rous sarcoma virus (RSV). The infected cells contain the RSV-transforming protein, pp60^{src}, which has characteristic tyrosine-specific protein kinase activity. As in other RSV-transformed cells, a 36,000-dalton protein is phosphorylated in RSV-infected A431 cells. Addition of epidermal growth factor (EGF) to the cells induces further phosphorylation of this protein. In contrast, this phosphoprotein is not detected in uninfected A431 cells, except when treated with EGF. Increased phosphorylation of the EGF receptor protein and of an 81,000-dalton cellular protein is dependent upon addition of EGF to the culture fluids, in both control and RSV-infected A431 cells. The results are discussed with reference to the similarities and differences between the tyrosine-specific protein kinases induced by RSV and activated by EGF.

The epidermal growth factor (EGF) receptor protein and the transforming proteins of at least four groups of RNA tumor viruses have associated protein kinase activity. These protein kinases are unusual in their strict specificity for tyrosine (1–10). This similarity and the fact that both EGF and tumor viruses can stimulate cell growth suggest that characterization of the protein kinases and identification of their substrates may help us understand the mechanism of growth control in normal and transformed cells.

The activity of tyrosine protein kinases can be measured in vitro with partially purified enzymes and added substrates, or in vivo where the action of the virus-associated or EGF-stimulated kinases may be observed as an increase in the net level of phosphotyrosine in cellular proteins. Cells transformed by Rous sarcoma virus, Abelson murine leukemia virus, feline sarcoma virus (Snyder-Theilen and Gardner-Arnstein strains), or the defective avian sarcoma viruses, Fujinami, PRCII, and Y73, have high levels of phosphotyrosine (6, 11–14). The effects of EGF on tyrosine phosphorylation are best demonstrated in a particular human tumor cell line, A431, which possesses a high level of EGF receptors. The level of phosphotyrosine increases three to four times within minutes of adding EGF (15). In the other cell types tested (3T3, HeLa), the increase is much lower, possibly because of the lower number of receptors in these cells. More detailed analysis, using a two-dimensional gel procedure that aids in the detection of proteins containing phosphotyrosine, has shown that the aforementioned viruses induce phosphorylation of members of the same set of cellular proteins, although quantitative differences exist (16, 17). At least one of the proteins phosphorylated in human A431 cells in response to EGF is homologous to one of the transformed cell phosphoproteins, as shown by peptide mapping of their nonphosphorylated forms (15). While we find its molecular weight to be 39,000, for clarity we will refer to this protein by its original molecular weight designation, 36K (16–21). One other protein (81K), whose phosphorylation on serine and tyrosine in A431 cells is EGF dependent (15), appears not to be phosphorylated in RSV-transformed mouse cells (17), but it is quite possible that the human protein is homologous to a mouse protein of different size or isoelectric point.

To explore further the similarities and differences between the effects mediated by EGF and by tumor viruses, we have derived a line (SRA431) of A431 cells infected with Rous sarcoma virus (RSV) and present here a preliminary characterization of its phosphoproteins.

MATERIALS AND METHODS

A431 cells were obtained from G. Todaro (National Institutes of Health, Bethesda, Md.) (22) and grown in Dulbecco's modified Eagle's medium (DME) with 10% calf serum. They were infected with Schmidt-Ruppin strain RSV, subgroup D (gift of L. Rorschneider [Fred Hutchinson Cancer Research Center, Seattle, Wash.]) by the method of Kawai (23). Subconfluent cells were overlaid with 1 ml of culture fluids from RSV-infected chick cells for 90 min at 37°C. The fluids were replaced with 1 ml 40% vol/vol polyethylene glycol 1500 (BDH Chemicals Ltd., Poole, England) for 1 min at 25°C. This was slowly diluted to a final volume of 4.5 ml and removed after 10 min. Surviving cells were grown in liquid culture for 17 d and then transferred to soft agar (24). Uninfected A431 cells, like most tumor cells, do grow in soft agar but poorly at low cell densities (24). Several particularly large colonies were picked from a 50-mm dish 4 wk after seeding with 10^3 infected cells. The infected cells were maintained under the same conditions as the parental cells.

Immune complex kinase assay for pp60^{src} was done as described (25). Extract from 1.6×10^4 cells was clarified and incubated with excess (5 μl) of normal rabbit or tumor-bearing baby rabbit serum at 0°C in 150 μl of buffer (25) containing 2 mM EDTA. Antibody was adsorbed to 1 mg of Pansorbin (Calbiochem-Behring Corp., San Diego, Calif.), and the complexes were washed repeatedly (25) before incubation with 5 μCi of [γ-^{32}P]ATP, in 10 μl of 20 mM

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PIPES, 10 mM $MnCl_2$, pH 7.0, at 37°C for 10 min. Immunoglobulin heavy chains were purified by gel electrophoresis and Cerenkov radiation was measured.

EGF binding was determined essentially as described (26, 27).

EGF additions to A431 and SRA431 cells were performed with subconfluent cultures, because we had found earlier that A431 cells only showed phosphorylation changes in response to EGF when cultured at low density (15). Cell cultures were labeled with 0.6 mCi of [^{32}P]orthophosphate (carrier-free, ICN Pharmaceuticals, Inc., Irvine, Calif.) per ml of phosphate-free DME containing 4% complete calf serum, for 12–18 h, to approach isotopic equilibrium. EGF (3.31 μM solution in 0.1% serum albumin) was added as required to 10 nM final concentration, for the last hour of labeling. The cultures were washed with cold Tris-buffered saline and rapidly prepared for phosphoamino acid analysis (11) or for two-dimensional gel electrophoresis (16). Two-dimensional gels were run as before (15–17, 28) with pH 6–8 ampholytes. They were incubated in alkali before autoradiography (16).

Membranes were partly purified from ^{32}P-labeled cells (mixed with unlabeled carrier cells) by a scaled-down version of a procedure (29) that yields preparations enriched for the EGF receptor (30). They were treated briefly with nucleases as for two-dimensional gel electrophoresis (16, 28), but the lyophilized samples were dissolved in electrophoresis sample buffer (see below) and run on a 15% acrylamide/0.087% bisacrylamide gel. For in vitro phosphorylation, membranes from ~8 × 10^5 unlabeled A431 cells were suspended in 5 μl of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonate, pH 7.4, and incubated with 5 μl of 20 mM PIPES, 10 mM $MnCl_2$, pH 7.0, containing 0 or 5 ng of EGF and 250 ng of serum albumin, at 0°C for 10 min. Then 5 μCi of [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.; 3,000 Ci/mmol) in 5 μl of the PIPES, $MnCl_2$ buffer were added. After 10 min at 0°C, the samples were adjusted to contain 2% SDS, 5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, and 10% glycerol, and one third of the reaction was analyzed by electrophoresis. Coomassie Blue staining showed proteins seen before in well-characterized membrane preparations (31).

Purification and peptide mapping of the 36K phosphoprotein from dishes of 1.3 × 10^5 cells labeled with 7.5 mCi of ^{32}P were described before (17).

RESULTS

Isolation and Characterization of RSV-infected A431 Cells

A431 cells were infected with RSV and cloned in soft agar. Four colonies were successfully grown and tested for pp60^{src} protein kinase activity. pp60^{src} was precipitated from cell extracts with serum from a rabbit bearing RSV-induced tumors, and transfer of phosphate to the immunoglobulin was used as a measure of protein kinase activity (25). The serum used precipitates the cellular homologue, pp60$^{c\text{-}src}$, inefficiently. On the basis of this assay, one cell clone contained high pp60^{src} activity (Table I, clone 6), another had somewhat less (clone 7), and two had none (clone 11 and one other clone not shown).

Upon precipitation of [^{35}S]methionine-labeled extracts, a 60,000 mol wt protein was readily detected in SRA431 clone 6 cells but not in uninfected controls (not shown). One-dimensional peptide mapping of the ^{35}S-labeled protein showed that partial *Staphylococcal* protease cleavage yields two major fragments characteristic of pp60^{src} (32) and different from those of pp60$^{c\text{-}src}$. We concluded that we had obtained at least one clone of A431 cells containing an active viral pp60^{src}. We will refer to this clone as "infected" rather than "transformed," since the parental cells are by definition already transformed, being established from a tumor.

TABLE I

Immune Complex Kinase Activity of Transformed A431 Cells

Cell line	cpm incorporated*	
	Non-immune serum	Immune serum
A431	26	45
SRA431, clone 6	23	1,423
SRA431, clone 7	23	1,121
SRA431, clone 11	24	48
SR3T3 (P1A5)‡	23	841
SRA431, clone 6		
Control§	35	1,152
+ EGF	38	1,124

* Immune complex kinase activity was measured. See Materials and Methods.

‡ Mouse 3T3 cells transformed with the same RSV used to infect SRA431 cells (gift of T. Patschinsky). The parental 3T3 cells have control levels of immune complex kinase activity.

§ In a separate experiment, cells were incubated with 0 or 10 nM EGF for 1 h before immunoprecipitation.

The infected cells also contained the RSV structural protein precursor, Pr76^{gag}, but the mature structural proteins were not detected. Culture fluids from SRA431 clone 6 cells were not infectious for chicken cells, in accord with the block in virus production found in other RSV-transformed mammalian cells (23, 33). In terms of morphology and growth rate in liquid culture, the infected cells were not significantly different from the parental A431 cells.

The number of specific EGF binding sites on SRA431 cells were estimated by standard procedures (26). We found 7 × 10^6 saturable EGF binding sites per A431 cell, in general agreement with published values (27), whereas there were 14 × 10^6 sites per SRA431 (clone 6) cell. We suspect that this difference is not because the SRA431 cells were infected but rather because the A431 cells used to derive the infected lines, and for control experiments, had not been cloned. Transformation by RSV does not inhibit EGF binding to mouse fibroblasts (34).

Both the infected and control A431 cells respond to the addition of EGF by rounding and by adhering less well to the substrate (35, and our data not shown). EGF is not mitogenic for A431 cells. On the contrary, recent reports (36, and Gill, G. N., and C. S. Lazar, manuscript submitted for publication.) have shown that low concentrations of EGF arrest the growth of A431 cells. We have repeated this observation with A431 cells and find that EGF also inhibits the growth of SRA431 cells (data not shown) and even appeared to be toxic.

Phosphoproteins of SRA431 Cells

The proteins of mammalian and avian cells transformed by RSV contain more phosphotyrosine than do the proteins of control cells (11). However, since the phosphotyrosine level in uninfected A431 cells is higher than in most nontransformed cells or other human tumor lines (15), there is only a small increase in level in the infected cells (Table II).

When A431 cells are treated with 10 nM EGF, the phosphotyrosine content of cell proteins quadruples within 1 min (15).

TABLE II

Phosphoamino Acid Analysis of Cellular Proteins

Cells	EGF	Relative proportion*		
		Phosphotyrosine	Phosphothreonine	Phosphoserine
	nM			
A431	0	0.18	8.04	91.78
	10	0.70	7.12	92.18
SRA431 (clone 6)	0	0.22	7.44	92.34
	10	0.97	8.10	90.92

* The radioactivity in each is expressed as a percentage of the total radioactivity in the three phosphoamino acids (11). The cells used were sister cultures of those prepared for the two-dimensional gels shown (Fig. 2).

Similarly, addition of EGF to SRA431 cells results in a threefold to fivefold increase in cellular phosphotyrosine, with the total level in stimulated SRA431 cells being higher than in stimulated A431 cells (Table II).

When SRA431 cells were incubated with ^{32}P and analyzed by two-dimensional gel electrophoresis, the 36K phosphoprotein was detected even in the absence of EGF (Fig. 1). The extent of phosphorylation depended on the particular clone of transformed A431 cells, being greater for the clone that had the highest pp60^{src} kinase activity when assayed in vitro (Table I). Thus, in human epidermoid cells, as well as a variety of other cells (16–20), transformation by RSV induces phosphorylation of a 36K cellular protein. The 81K phosphoprotein was not detected in any of the clones of infected A431 cells (Fig. 1).

Treatment of SRA431 cells with EGF induces increased phosphorylation of a number of proteins (some of which are indicated in Fig. 2 *D*). Not all of these changes were found in every experiment; however, when a particular phosphoprotein was found at increased levels in EGF-treated SRA431 cells, it was also found in parallel cultures of EGF-treated A431 cells (Fig. 2 *B*). Increased phosphorylation of two SRA431 cell proteins was detected after EGF treatment in every experiment. These were the 36K and 81K phosphoproteins observed before (15; Fig. 2 *B* and *D*, large arrowheads).

Increased phosphorylation of SRA431 cell 36K protein after EGF treatment was also detected by two-dimensional gel electrophoresis of proteins labeled with [^{35}S]methionine (Fig. 3). The phosphorylated form of the 36K protein was located by autoradiography of mixed ^{32}P- and ^{35}S-labeled proteins (Fig. 3 *A*). The nonphosphorylated form lies beyond the basic end of the gel, to the right. Since most of the [^{35}S]methionine-labeled proteins are not phosphoproteins and do not alter in amount after brief exposure to EGF, it is easy to observe increased intensity of this spot relative to other spots, implying increased phosphorylation of the 36K protein (Fig. 3 *B–D*). Densitometry of autoradiographs of ^{32}P-labeled proteins suggested that the final relative level of phosphorylation was greater with the combined actions of EGF and infection than with either agent alone. This implies that stimulation of the EGF-dependent kinase does not interfere with the action of pp60^{src}, and vice versa. Event though the effect of infection by RSV was much smaller than that of EGF when measured by the increment in the cellular level of phosphotyrosine (Table II), the effect of infection on phosphorylation of the 36K protein was about double the effect of EGF alone. Presumably, the high level of phosphotyrosine in A431 cells is contributed partly by phosphoproteins that are not detectable, for a variety of reasons, by the two-dimensional gel procedure (16).

Infection of A431 cells does not interfere with the EGF-dependent phosphorylation of the 81K protein. The 81K phosphoprotein is readily detected at two to three times higher levels in SRA431 cells than in A431 cells after EGF addition (Fig. 2), in keeping with the higher EGF binding in these cells.

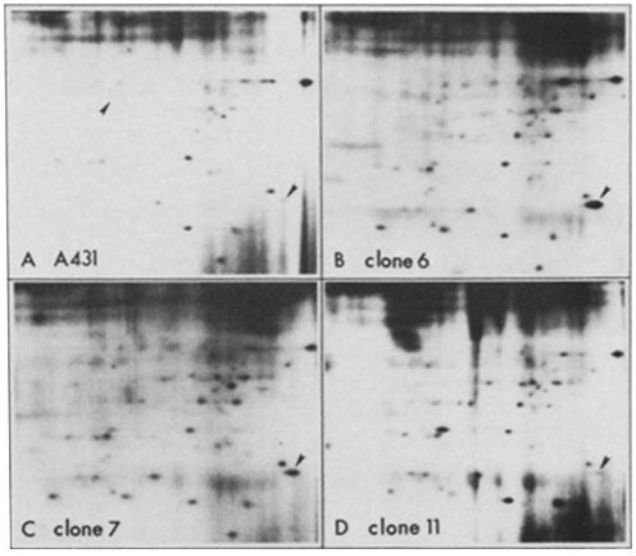


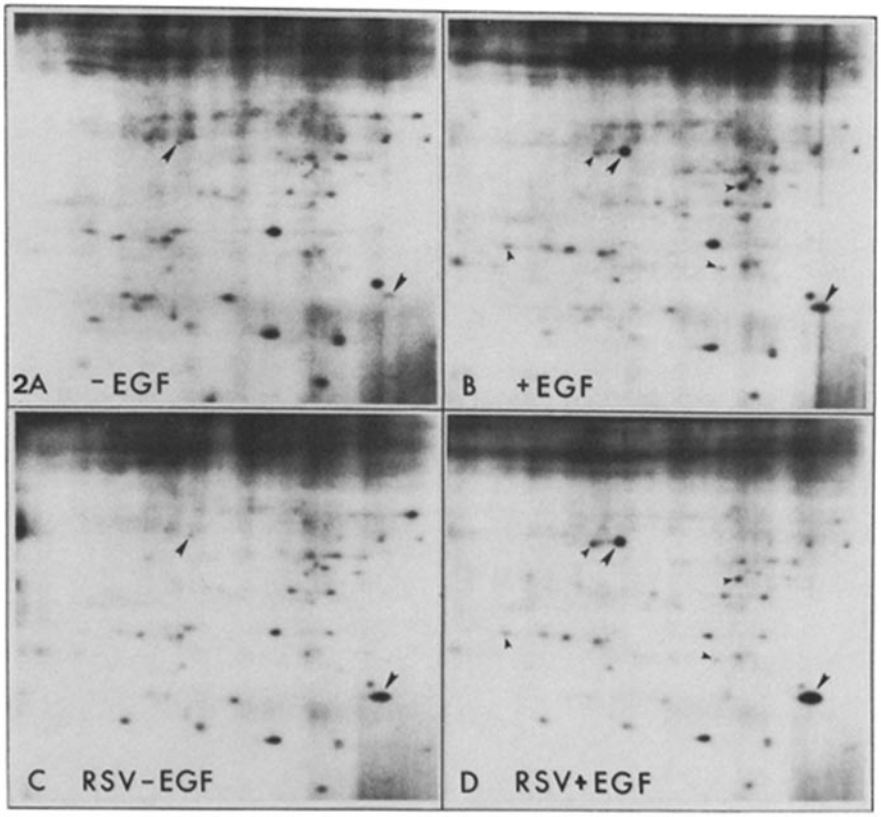
FIGURE 1 Phosphoproteins of control and infected A431 cells. Parallel cultures of control (*A*), SRA431 clone 6 (*B*), clone 7 (*C*), or clone 11 (*D*) cells were labeled for a total of 14 h with [^{32}P]-orthophosphate. No EGF was added. Cultures were prepared, and samples equivalent to ~3 × 10^4 cells were analyzed by two-dimensional gel electrophoresis. Alkali-treated gels were exposed for 3–5 d with intensifying screens. The positions of 81K and 36K phosphoproteins are indicated by arrowheads.

Phosphorylation of pp60^{src} and of the EGF Receptor

The RSV transforming protein, pp60^{src}, contains phosphotyrosine (2), so we tested whether EGF could induce increased phosphorylation of pp60^{src}. We purified pp60^{src} from ^{32}P-labeled control and EGF-treated SRA431 cells by immunoprecipitation and gel electrophoresis. Similar amounts of radioactivity were recovered (301 and 225 cpm, respectively). Moreover, partial proteolysis showed that the distribution of phosphate between the N-terminal (phosphoserine) and C-terminal (phosphotyrosine) regions was unaltered (data not shown). The protein kinase activity of pp60^{src} isolated from unlabeled SRA431 cells was the same whether or not the cells had been incubated with EGF (Table I).

The EGF receptor of A431 cells is a 150,000–170,000 mol wt glycoprotein (37–39), containing many phosphorylation sites in vivo (15), which does not focus on two-dimensional gels, although it can be purified from ^{32}P-labeled A431 cells by immunoprecipitation and SDS gel electrophoresis. We found that the receptor contained phosphoserine, phosphothreonine, and phosphotyrosine residues and that phosphorylation of all sites in the receptor increased two to three times after EGF treatment of the cells (15). To test whether transformation by RSV affected the phosphorylation of the receptor, we partially purified membranes from ^{32}P-labeled SRA431 cells and separated the proteins on SDS gels (Fig. 4). A phosphoprotein, containing relatively alkali-stable phosphate, was found that has the same mobility, on both 15 and 7.5% polyacrylamide gels, as the major product of in vitro phosphorylation of membranes prepared from unlabeled A431 cells (Fig. 4 *A*). Cohen et al. (38) have shown that this phosphoprotein is the EGF receptor. Phosphorylation of this membrane protein was increased two to three times by EGF treatment of A431 or SRA431 cells (Fig. 4 *B*).

We were surprised to find that the 155,000 mol wt (155K) phosphoprotein could also be detected by SDS gel electrophoresis of total cellular phosphoprotein, provided the gels were treated with alkali before autoradiography (Fig. 4 *C*). This analysis also showed EGF-dependent phosphorylation of the receptor. The somewhat greater amount of phosphate in the EGF receptor of SRA431 cells, relative to A431 cells, may reflect the higher number of EGF binding sites in the transformed clone.



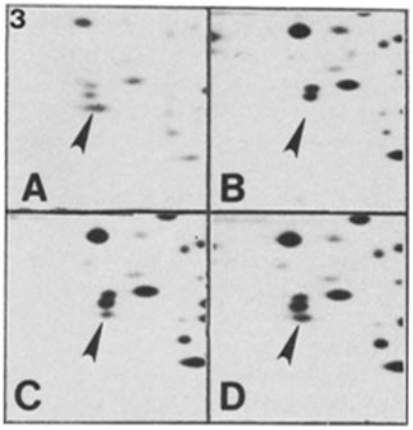


FIGURE 2 Effect of EGF on phosphoproteins of control and infected A431 cells. Parallel cultures of A431 (*A* and *B*) and SRA431 clone 6 (*C* and *D*) cells were labeled for 16 h with [^{32}P]orthophosphate. EGF (10 nM final concentration) was added to one culture of each (*B* and *D*) for the final hour of labeling. Cultures were analyzed as before (see Fig. 1). All exposures were for 3 d.

FIGURE 3 Effect of EGF on proteins of control and RSV-infected A431 cells. Parallel cultures of A431 and SRA431 cells were labeled with ^{32}P or [^{35}S]methionine and treated with EGF. Cultures were lysed and samples analyzed on two-dimensional gels, which were not incubated in alkali. (*A*) Extract of 3.35×10^4 ^{35}S-labeled SRA431 cells (EGF-treated) (3.67×10^6 acid-precipitable cpm) mixed with 3.35×10^4 ^{32}P-labeled SRA431 cells (EGF-treated). Part of a 48-h autoradiograph is shown. The arrowheads point to the 36K phosphoprotein, identified by exposing the same gel with aluminum foil blocking the ^{35}S radioactivity (16). (*B–D*) ^{35}S-labeled cells, gels impregnated with 2,5-diphenyloxazole and exposed so that the product of time exposed (min) and acid-precipitable cpm loaded was 3×10^8. (*B*) A431 cells (no EGF). (*C*) SRA431 cells (no EGF). (*D*) SRA431 cells (10 nM EGF).

Phosphopeptides of 36K phosphoprotein

To examine the possibility that a common kinase phosphorylates the 36K protein, we tested whether EGF and RSV induced phosphorylation of different sites in this protein. The major phosphopeptides of the mouse 36K protein phosphorylated in RSV- or Abelson murine leukemia virus–transformed cells are quite different from those of the human 36K protein phosphorylated in response to EGF, even though the two proteins appear to be quite similar in primary structure (15). However, the 36K protein from SRA431 cells contained a major phosphotyrosine-containing peptide (Fig. 5*A*) similar in mobility to that found in 36K protein from EGF-treated A431 cells (15). Addition of EGF to SRA431 cells did not induce the phosphorylation of additional peptides (Fig. 5*B*), suggesting that in these cells RSV and EGF induce phosphorylation of the same or closely positioned tyrosine residues. Thus, we cannot exclude the possibility that RSV and EGF both induce the phosphorylation of the 36K protein by activating a third tyrosine-specific protein kinase.

DISCUSSION

We have shown that A431 human tumor cells infected with RSV, and containing a functional pp60^{src} transforming protein, are able to bind and respond to EGF much as do cells of the parental line. However, the transformed cells contain the phosphorylated form of a 36K protein that is normally detected in the parental A431 cells only when they are treated with EGF. Treatment of the infected cells with EGF induces increased phosphorylation of this 36K protein and of two other proteins, an 81K protein and the 155K EGF receptor protein. The 81K protein is not normally phosphorylated in either control or transformed A431 cells but is phosphorylated after EGF addition. The EGF receptor is normally phosphorylated in both cell types, but it is more highly phosphorylated after EGF addition.

The isolation of A431 cells infected with RSV has enabled us to examine some questions regarding the modes of action of pp60^{src} and EGF. Our experiments with in vivo labeling show that the effects of RSV and of EGF are not competitive, implying that these agents have different routes of action, or that steps common to both pathways are not operating at maximum capacity with either pathway alone. The intriguing possibility of chains of tyrosine protein kinases, activating each other in sequence, has recently been raised (40). The proximal protein kinase for phosphorylation of the 81K protein could well be the EGF receptor–associated kinase itself, since SRA431 cells contain approximately twice as many receptor molecules and give a 1.5- to 3-fold higher level of phosphory-

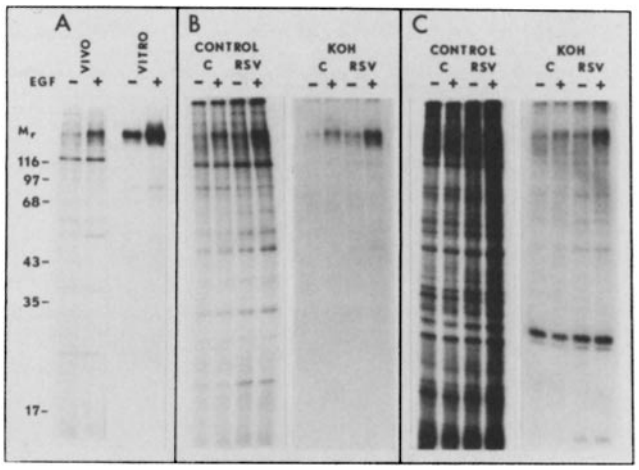


FIGURE 4 Effect of EGF on membrane phosphoproteins. Duplicate cultures containing ~10^6 A431 and SRA431 clone 6 cells were labeled for 14 h with 1.2 mCi/ml of [^{32}P]orthophosphate. EGF was added as required, and membranes were purified (Materials and Methods). (*A*) Membrane fractions from ^{32}P-labeled SRA431 cells, treated in vivo with 0 or 10 nM EGF, were electrophoresed beside membranes from unlabeled A431 cells, which had been phosphorylated in vitro in the absence or presence of EGF (30; Materials and Methods). The gel was autoradiographed for 5 h. (*B*) Membrane fractions from 8.3 × 10^4 A431 cells (*C* [under *control* and *KOH*]) or SRA431 cells (*RSV*), labeled and treated with EGF in vivo, were analyzed. The dried gel was autoradiographed for 18 h (*control*), then incubated in alkali (18) and autoradiographed for 40 h (*KOH*). (*C*) Complete cell samples (8.3 × 10^3 cells) were analyzed as in *B*. The molecular weight scale applies to *A*; the gels in *B* and *C* were electrophoresed slightly longer.

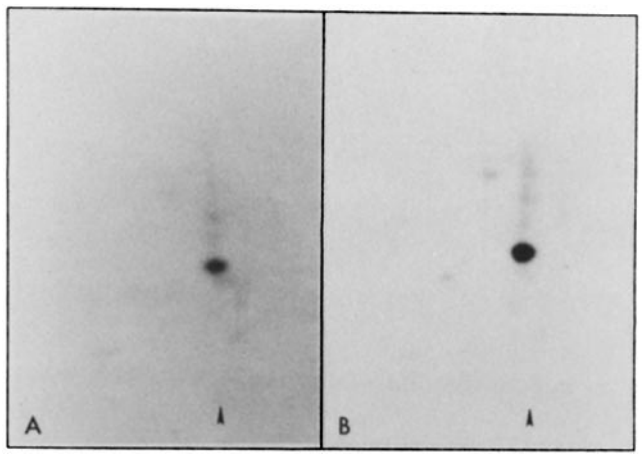


FIGURE 5 Phosphopeptides of 36K phosphoprotein. Tryptic digests were analyzed by two-dimensional thin-layer electrophoresis (anode at left) and chromatography, and autoradiographed with intensifying screens. The arrowheads mark the points of sample application. The major radioactive spot contains only phosphotyrosine; the phosphoserine is labile during the preparation for peptide mapping. (*A*) 36K protein from untreated SRA431 cells (80 cpm; 8-d exposure). (*B*) 36K protein from EGF-treated SRA431 cells (240 cpm; 4-d exposure).

lated 81K protein upon EGF addition than do the control A431 cells. Clearly, pp60^{src} does not activate this EGF-dependent kinase since only EGF induces phosphorylation of the 81K protein. The EGF-dependent kinase does not appear to activate pp60^{src}, since we have found that the activity of pp60$^{c\text{-}src}$ of normal A431 cells (data not shown) and the activity and phosphorylation of pp60^{src} of SRA431 cells are unaltered by EGF.

Both systems induce the phosphorylation of the 36K protein, apparently at a tyrosine in the same tryptic peptide, although it is possible that this peptide contains more than one tyrosine. Erikson and Erikson (20) showed that pp60^{src}, purified to apparent homogeneity, could phosphorylate purified 36K protein, in vitro, suggesting that pp60^{src} could phosphorylate the 36K protein directly in vivo. However, it is difficult to exclude the possibility that pp60^{src} could activate another protein kinase with specificity for the same tyrosine of the 36K protein. It is not yet known whether the 36K protein can be phosphorylated by the EGF receptor–associated kinase in vitro. The observation that the receptor–associated kinase can phosphorylate sera raised against RSV (41, 42), which are also substrates for pp60^{src}, shows that both kinases can recognize the same substrates.

Both pp60$^{c\text{-}src}$ and the EGF receptor–associated kinase are found in membrane preparations of A431 cells (30, 41). Addition of EGF and [γ-^{32}P]ATP to unlabeled membranes allows phosphorylation of a protein of ~80,000 mol wt (39); but this protein differs from the 81K phosphoprotein observed in vivo in its mobility on two-dimensional gels (not shown). Indeed, this latter phosphoprotein was not detected in membranes partially purified from ^{32}P-labeled, EGF-treated cells (not shown).

Further analysis of this system awaits the identification of potential intermediary kinases and further characterization of the substrates. The possible role of the 36K and 81K proteins in mediating the biological effects of EGF in other cell types remains to be explored.

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