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Tyrosine Phosphorylation of Specific Proteins After Mitogen Stimulation of Chicken Embryo Fibroblasts

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We found that stimulation of density-inhibited chicken embryo fibroblasts with serum, epidermal growth factor (EGF), platelet-derived growth factor, (PDGF), or multiplication-stimulating activity (MSA) leads to an increase in tyrosine phosphorylation of proteins in the region of M_r 40,000 (40K) to 42K. The increase in tyrosine phosphorylation after serum or EGF stimulation was transient, reaching a maximum at about 5 min and then declining. By fine-resolution analysis of proteins separated on sodium dodecyl sulfate-polyacrylamide gels, we found that after EGF stimulation, the major increase in phosphotyrosine content was in a 42K M_r protein, with a smaller increase in a 40K M_r protein. The increased phosphorylation in the 40K to 42K M_r region accounted for almost all of the increase in phosphotyrosine observed in these cells. These phosphotyrosine-containing proteins were different from the major phosphotyrosine-containing protein of Rous sarcoma virus-transformed chicken embryo fibroblasts, which migrates at an approximate M_r of 36K. Increased tyrosine phosphorylation of proteins of similar M_r was found in 3T3 cells treated with EGF, but not in NR-6 cells, which lack detectable EGF receptors. It is possible that the 40K to 42K M_r phosphotyrosine-containing proteins are involved in the integration of the biological response to a number of different growth factors.

We have shown previously that the transformation of chicken embryo fibroblasts (CEF) by Rous sarcoma virus (RSV) leads to the phosphorylation on tyrosine of a large number of cellular proteins (32). Since $pp60^{src}$, the transforming protein of RSV, has been shown to be a tyrosine-specific protein kinase (12, 13, 20, 21, 28), the cellular proteins which become phosphorylated on tyrosine in transformed cells are candidate cellular targets for $pp60^{src}$. Other investigators have also found, by two-dimensional gel electrophoresis, a number of cellular proteins which become phosphorylated on tyrosine after virus transformation (14, 15, 37, 38). In addition, immunoprecipitation experiments have revealed tyrosine phosphorylation in $pp60^{src}$ (21, 28), vinculin (44), and a 50,000 (50K) M_r protein which coimmunoprecipitates with $pp60^{src}$ (7, 35). The functional identity of these tyrosine-phosphorylated proteins is still unknown, except for $pp60^{src}$ and vinculin, the latter being a cytoskeletal protein which may be involved in the anchorage of microfilaments to the plasma membrane (23).

In addition, we have shown that in normal, growing CEF there are at least two proteins with M_rs of approximately 40K and 42K which are phosphorylated on tyrosine (32). Presumably these are targets for a normal cellular tyrosine-specific protein kinase. It remains to be determined which, if any, of these phosphorylated targets play a physiologically important role in either normal cell function or the transformation process by RSV. In particular, it is not known whether any of these phosphoproteins play a role in the control of growth.

Recent reports demonstrate that several polypeptide growth factors can stimulate tyrosine phosphorylation in a number of cell types (18, 27, 47). This raises the possibility that a similar mechanism may underlie both mitogen- and tumor virus-induced growth stimulation. If such is the case, it might be expected that the two processes would share one or more substrates. In fact, it has been shown that the major cellular protein which is phosphorylated in RSV-transformed cells, the "36K" protein originally described by Radke et al., (37, 38), is also phosphorylated on tyrosine after epidermal growth factor (EGF) stimulation of A431 cells, a human carcinoma cell line (15, 19). The A431 cells may, however, represent an atypical situation, since it has been shown that with these cells, EGF is not mitogenic but is actually growth inhibitory (2, 24).

We examined CEF and 3T3 cells after stimula-

tion by serum or various mitogens to identify proteins which become phosphorylated on tyrosine. We found that in response to such stimulation there is an apparent increase in the tyrosine phosphorylation of proteins in the region of M_r 40K to 42K. It is possible that the cellular proteins phosphorylated after mitogen stimulation play a role in the biological response to a variety of mitogenic signals.

MATERIALS AND METHODS

Cells and cell culture. CEF primary cultures were prepared from the body walls of 10 to 11-day-old embryos by standard techniques (48). Secondary cultures were plated at high density in Dulbecco modified Eagle medium containing 1% calf serum and 1% heat-inactivated chicken serum. The cultures were at confluence 2 to 3 days after plating. Infection of secondary CEF with Schmidt-Ruppin A RSV was carried out as previously described (1, 32).

The Swiss mouse 3T3 cell line and its EGF-receptor-less mutant, NR-6 (36), were obtained from Harvey Herschman, University of California, Los Angeles, and were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Radiolabeling. After reaching confluence, the culture fluid was replaced with 2 ml of phosphate-free Dulbecco modified Eagle medium; 3 mCi of $^{32}P_i$ per ml was added to each 60-mm dish, and the cells were incubated for a further 6 to 8 h. Serum or growth factors were then added, and the cells were incubated for various times. At the end of the incubation period, the cells were washed once in cold phosphate-buffered saline and then rapidly lysed in 500 μl of hot Laemmli electrophoresis sample buffer (30) as described earlier (32).

Electrophoresis and phosphoamino acid analysis. The cell extracts were electrophoresed on 10% polyacrylamide tube gels, the gels were sliced into 30 slices, and the proteins were eluted from each slice as described previously (32). The proteins were then precipitated in trichloroacetic acid along with 400 μg of immunoglobulin G. After the trichloroacetic acid pellets were washed in ethanol and ethanol-ether (1:1) at −20°C, they were dissolved directly in 1 ml of distilled hydrochloric acid and hydrolyzed for 2 h at 110°C under nitrogen. After removal of the hydrochloric acid under reduced pressure, the hydrolysates were analyzed for their phosphoamino acid content by high-voltage paper electrophoresis at pH 1.9 and 3.5 as described earlier (32), except for the following modification. In our previous analysis we carried out the electrophoresis via a two-stage, one-dimensional separation to accommodate a very large number of samples. The method suffered somewhat from the trailing of radioactivity from the highly radioactive phosphoserine and phosphothreonine into the much less radioactive phosphotyrosine. Therefore, in the present study, all analyses were by a two-dimensional analysis which gives the cleanest separation of the phosphoamino acids (Fig. 1C). However, to maximize the number of samples which could be run together, the second electrophoresis at pH 3.5 was carried out on only the phosphothreonine-phosphotyrosine spot (Fig. 1B). To accomplish this, the phosphothreonine-phosphotyrosine spot was localized autoradiographically on the pH 1.9 electropherogram. The area containing these two phosphoamino acids was cut out, sewn onto another Whatman 1MM paper, and electrophoresed at pH 3.5.

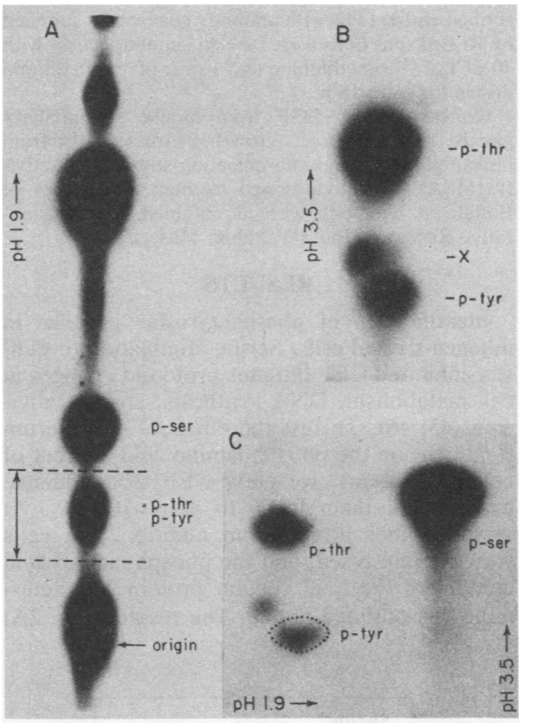

FIG. 1. Two-dimensional separation of phosphoamino acids by high-voltage paper electrophoresis. (A) First separation at pH 1.9 (B) Region in panel A containing the phosphotyrosine (p-tyr) and phosphothreonine (p-thr), shown by the dotted lines, run at pH 3.5 at a 90° angle to the first dimension. This is the method used for all of the analyses reported in this paper. (C) Separation when the phosphoserine (p-ser) was included with the other two phosphoamino acids in the second electrophoretic run.

The background in this separation method is determined by the degree of streaking from phosphothreonine. This was corrected for by subtracting the counts from an area of the electropherogram that is representative of the degree of streaking from the counts associated with the phosphotyrosine spot. Aside from the three phosphoamino acids, our two-dimensional analysis revealed at least two more phosphorylated species of unknown identity that were generated from some areas of the polyacrylamide gel. In all of our separations, however, the ninhydrin-stained phosphotyrosine standards were resolvable from these two phosphorylated molecules. Finally, the radioactivity associated with each phosphoamino acid was determined by liquid scintillation counting of each ninhydrin-stained spot. Quantitation of phosphoserine from either pH 1.9 or pH 3.5 electropherograms gave equivalent results.

Immunoprecipitation. Immunoprecipitation of the "36K" protein was carried out essentially as de-

scribed earlier (34), with antibody generously supplied by R. Erikson. Cells were labeled simultaneously with 70 μCi of [^{35}S]methionine and 1 mCi of $^{32}P_i$ in 35-mm dishes for 16 to 18 h.

Growth factors. EGF from mouse submaxillary glands, platelet-derived growth factor (PDGF) from human platelets, and multiplication-stimulating activity (MSA) from conditioned medium of cultures of Buffalo rat liver cells were all obtained from Collaborative Research, Inc. (Waltham, Mass.).

RESULTS

Identification of phosphotyrosine proteins in mitogen-treated cells. Serum stimulation of density-inhibited CEF initiates profound changes in cell metabolism, DNA synthesis, and cell division (45, 46). To test the effect of such serum treatment on the phosphoamino acid content of cellular proteins, we grew CEF to confluence and labeled them for 6 to 8 h with $^{32}P_i$. At various times after serum addition, the cells were rapidly lysed, and the phosphoamino acid content of the total cellular protein was determined as outlined above. The results (Fig. 2A) showed an immediate increase in tyrosine phosphorylation after serum addition, amounting to a 1.4- to 1.7-fold increase. However, the increase in phosphotyrosine was transitory, rapidly reaching a maximum level at about 5 min and then declining. By 180 min after the initial serum addition, the phosphotyrosine content had fallen to the prestimulated levels. The rate of 2-deoxyglucose uptake was followed in parallel culture dishes (Fig. 2A). As has been reported previously (45, 46), an increase in transport rate could be detected within minutes after serum stimulation. However, in contrast to the phosphotyrosine content, the elevated transport rate was maintained for several hours.

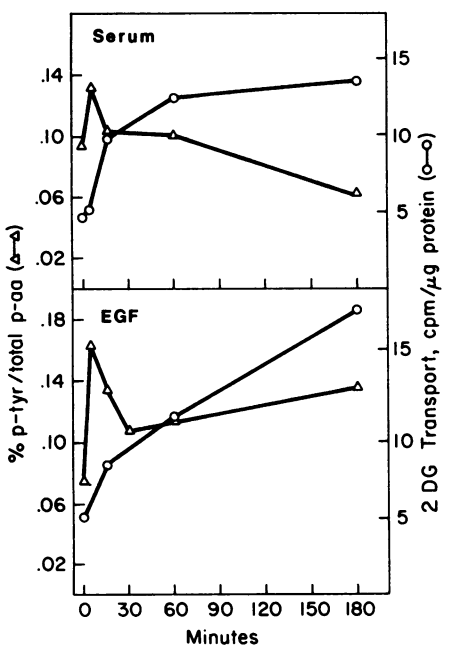

FIG. 2. Time course of stimulation of phosphotyrosine (p-tyr) content of CEF treated with serum or EGF. Density-inhibited CEF labeled with 1 mCi of $^{32}P_i$ per 35-mm dish were treated with chicken serum (10%) or EGF (500 ng/ml). At various times the cells were lysed in hot sodium dodecyl sulfate buffer and precipitated with trichloroacetic acid, and the phosphoamino acid (p-aa) content was determined as described in the text. Parallel unlabeled cultures were used to determine the rate of 2-deoxyglucose (2 DG) transport as previously described (48).

EGF has been found to be a potent mitogen for a variety of cell types (9). It has also been found that in the A431 human carcinoma cell line, the addition of EGF results in the stimulation of tyrosine phosphorylation (15, 19, 27, 47). We found, as with serum, that when EGF was added to quiescent CEF, there was a transient increase in the overall phosphotyrosine content (Fig. 2B). For EGF the increase in phosphotyrosine was about twofold. As an indication of the biological responsiveness of these cells to EGF, we found an increase in the transport rate of 2-deoxyglucose that was comparable to the increases found with serum addition (Fig. 2) (3). The CEF were also stimulated to increase their rate of DNA synthesis fivefold (measured by [^{3}H]thymidine incorporation 13 h after stimulation) (data not shown).

To identify the proteins which were being phosphorylated in response to the serum or EGF, we analyzed ^{32}P-labeled extracts by electrophoresis on polyacrylamide tube gels as described above. The autoradiograms for the analysis of CEF and EGF-stimulated CEF in Fig. 3 show only the exposures for phosphotyrosine and phosphothreonine, although autoradiographic exposures for phosphoserine were also carried out for each analysis. The autoradiogram of the control CEF revealed a significant amount of phosphotyrosine in slices 12 to 13 and slices 17 to 18. Visually, one can see a dramatic increase in phosphotyrosine in gel slice 16 within 5 min of EGF addition to CEF. This peak of phosphotyrosine migrated slightly faster than the 43K M_r marker protein. Quantitation of the radioactivity associated with each of the phosphoamino acid spots was carried out as previously described (32) by cutting out the ninhydrin spots and counting by liquid scintillation spectrometry. The results presented in Fig. 4 give the percentage of phosphotyrosine contained in each gel slice with respect to the total phosphoamino acid content of the entire gel. This way of expressing the data controls for possible variations in ^{32}P pool size and specific activity. The

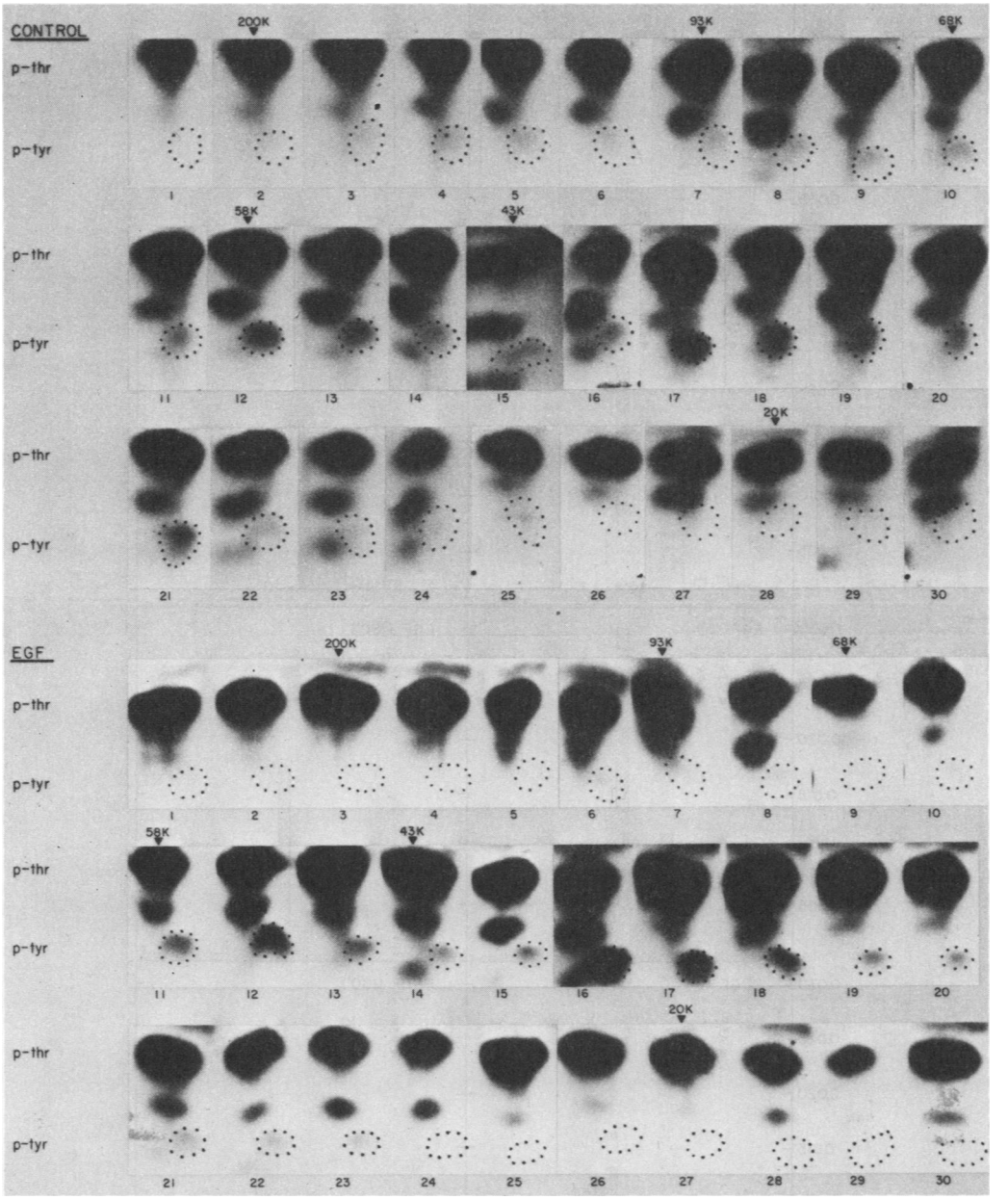

FIG. 3. Autoradiograms of phosphoamino acid analysis of gel-separated phosphoproteins of CEF either untreated or treated with EGF (500 ng/ml) for 5 min. Gel slice numbers are indicated beneath each row of autoradiograms. The positions on the gel of dansylated proteins used as internal molecular weight markers are indicated by arrows.

density-inhibited control cells exhibited three main peaks of phosphotyrosine: one migrating slightly ahead of the 58 K M_r marker protein, one migrating with the 43K M_r marker, and the major peak migrating slightly ahead of the 43K M_r marker. In the case of both serum and EGF addition, there was a rapid tyrosine phosphorylation of protein in the region of the gel corresponding to M_rs of around 40K to 42K. The increase in phosphotyrosine in this region after serum and EGF stimulation amounted to 2.8- and 3.5-fold, respectively. Although there may also have been modest changes in phosphorylations on serine and threonine, the large number

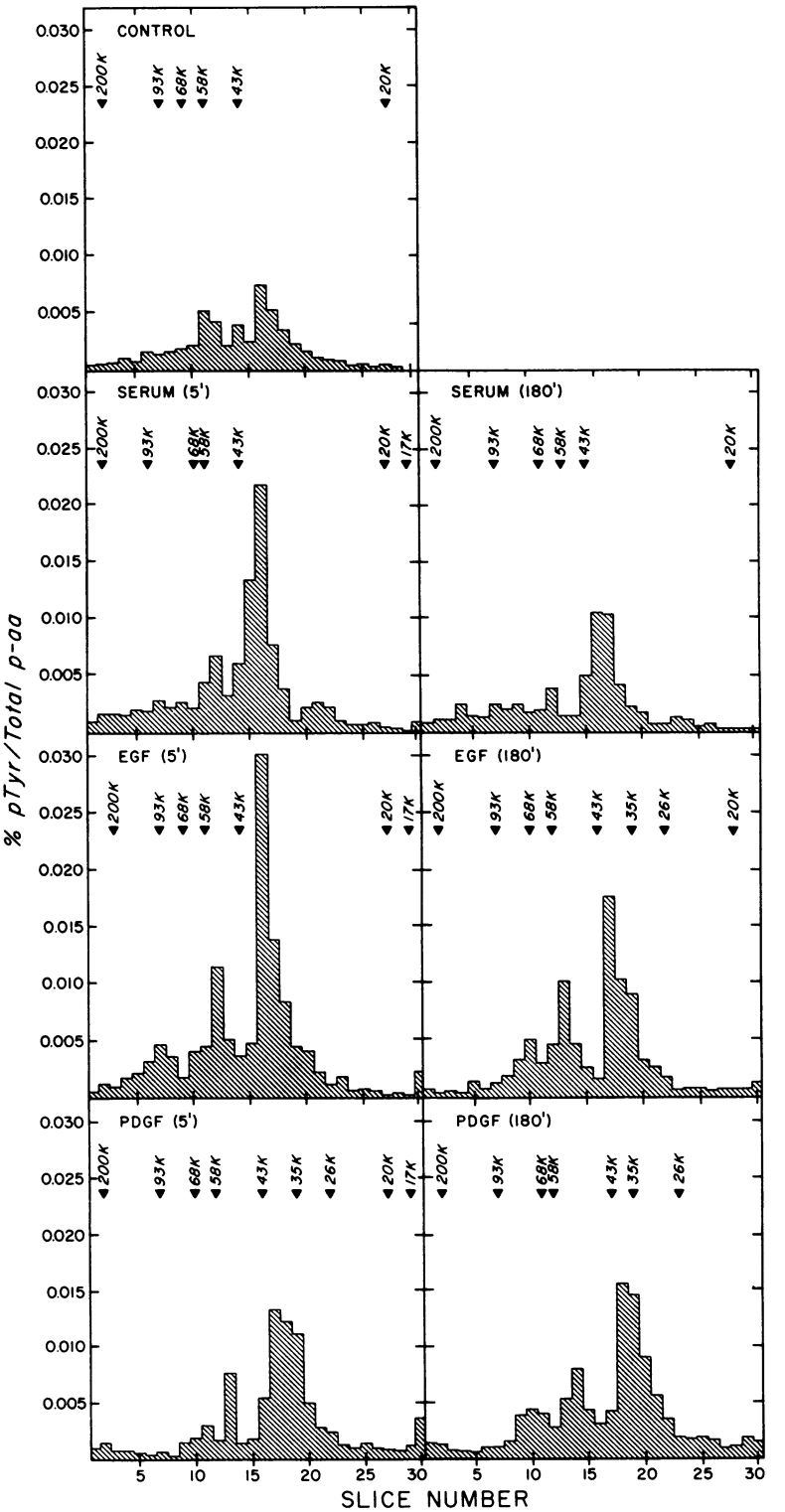

FIG. 4. Quantitative distribution of phosphotyrosine (pTyr) across polyacrylamide gels used to separate whole cell lysates of CEF. The individual ninhydrin-stained spots for phosphotyrosine, phosphothreonine, and phosphoserine from the two-dimensional, high-voltage electrophoresis analysis were cut and counted by liquid scintillation spectrometry. These are presented as the percentage of phosphotyrosine contained in each gel slice with respect to the total phosphoamino acid (p-aa) content of the entire gel. Density-inhibited CEF labeled with 3 mCi of $^{32}P_i$ per 60-mm dish were treated with either chicken serum (10% final concentration), EGF (500 ng/ml), or PDGF (500 ng/ml) for the times (in minutes) indicated within parentheses. The positions on the gel of dansylated molecular weight marker proteins are indicated by arrows.

of proteins containing these phosphoamino acids prevented any definitive conclusions (data not shown). In the experiment shown, the cells had been treated with 500 ng of EGF per ml. The results obtained with 50 ng/ml were the same except that the increase in phosphotyrosine was approximately one-half as great (data not shown). By 180 min, the tyrosine phosphorylation in this region of the gel had decreased, which is in agreement with the results of the total phosphotyrosine analysis shown in Fig. 2.

There was no comparable increase in tyrosine phosphorylation of either of the other two phosphotyrosine peaks detected in control CEF in response to serum or EGF. Furthermore, there did not appear to be other major increases in phosphotyrosine content in other regions of the gel, although slight increases in phosphotyrosine were sometimes seen in the M_r region around 100K. Thus, the increase noted in the 40K to 42K M_r region accounted for almost all of the total phosphotyrosine increase after serum or EGF stimulation.

PDGF is a major mitogenic component known to be present in serum (29, 40, 41). Whereas treatment of density-inhibited CEF with PDGF did not produce a consistent increase in total phosphotyrosine content (data not shown), the analysis of sodium dodecyl sulfate-polyacrylamide gels revealed an increase in the amount of tyrosine phosphorylation in the 40K to 42K M_r region (Fig. 4). The stimulation of the peak fraction appeared to be somewhat smaller than the effect with either serum or EGF. On the other hand, the phosphorylation in this region was more persistent, with no apparent decrease in phosphotyrosine even after 180 min of treatment with PDGF. As noted above, the phosphorylation initiated by serum and EGF was transitory.

MSA, a somatomedin-like growth factor consisting of a family of small polypeptides (16, 17), stimulates DNA synthesis and glucose transport in CEF (46) by binding to specific cell surface receptors (39). The pattern of tyrosine phosphorylation after MSA treatment appears to be quite similar to that seen for serum- and EGF-stimulated cells (Fig. 5), with an approximately 2.7-fold stimulation of phosphotyrosine in the 40K to 42K M_r region of the gel.

Relationship to the 36K target of pp60^{src}. An increase in tyrosine phosphorylation in response to EGF has been reported for a human carcinoma cell line, A431, which possesses an abnormally large number of EGF receptors. It has also been shown that the major tyrosine-phosphorylated protein after EGF treatment of these cells is the same major cellular protein which is phosphorylated on tyrosine after viral transformation by RSV (15, 19, 27). This is the "36K"

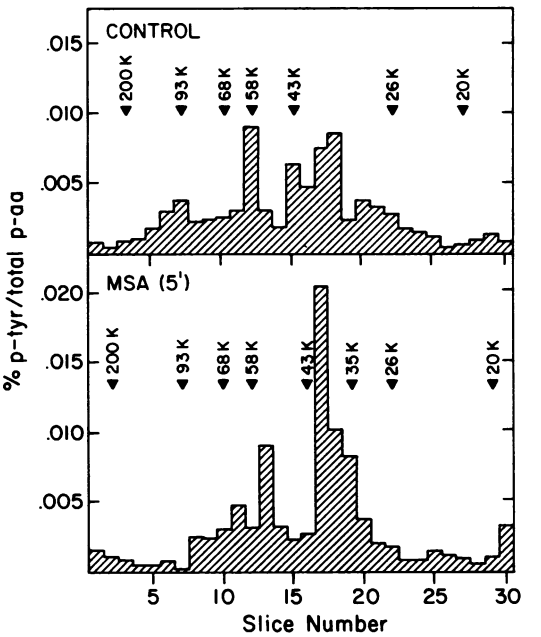

FIG. 5. Quantitative distribution of phosphotyrosine (p-tyr) across polyacrylamide gels used to separate whole cell lysates of CEF treated with MSA. Cells labeled with $^{32}P_i$ were treated for 5 min with MSA (500 ng/ml). The results were obtained and presented as outlined in the legend to Fig. 4.

protein originally described by Radke and Martin (37, 38). A comparison of the analysis of mitogen-treated CEF (Fig. 4 and 5) with our earlier reported analysis of RSV-transformed CEF (32) indicated that the major phosphotyrosine peaks in the mitogen-stimulated cells were not identical to the 36K protein. We demonstrate below that this is the case.

Utilizing antibody directed against the 36K protein, we were able to immunoprecipitate the protein from lysates of CEF stimulated for various times with EGF. However, we were unable to detect any increase in tyrosine phosphorylation of this protein in response to EGF (Fig. 6). On the other hand, the increased phosphorylation state of the 36K protein was readily detectable in RSV-transformed cells, even in cells, such as those infected with *ts*GI201 at 36°C or *ts*GI253 at 42°C (Fig. 6), which are only minimally transformed (1, 4, 34, 49). Thus, by immunological critiera, the mitogens do not stimulate detectable phosphorylation of the 36K protein.

It remained possible, however, that a small increase in phosphorylation of the 36K protein might go undetected because of the presence of a large excess of unphosphorylated protein, or because of the action of phosphatases which might not have been fully inhibited by the zinc

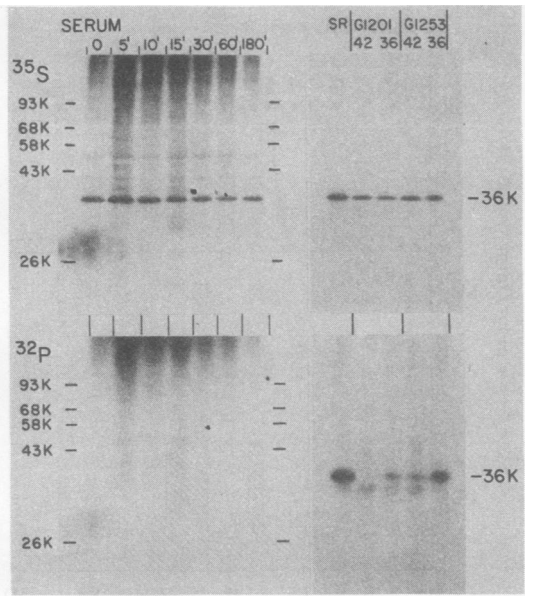

FIG. 6. Phosphorylation of the 36K protein in serum-stimulated and RSV-transformed CEF. The procedure for immunoprecipitation of the 36K protein with rabbit anti-chicken 36K antiserum and the electrophoresis on 12% polyacrylamide slab gels have been previously described (34). The upper panel shows the film exposure resulting from the incorporation of [^{35}S]methionine, and the lower panel shows the exposure due to $^{32}P_i$. Both exposures are from the same gel.

we included in our buffers (6, 22), or for both reasons. To resolve this question, we carried out fine-resolution analysis of the region from about 30K to 43K M_r on polyacrylamide gels of EGF-stimulated and RSV-transformed CEF, either alone or in combination. The finer resolution was accomplished by cutting this region into 1-mm slices rather than the 4-mm slices of the analyses shown in Fig. 4 and 5. The results (Fig. 7) clearly demonstate that the major tyrosine-phosphorylated protein in this region from EGF-stimulated CEF migrated differently on sodium dodecyl sulfate-polyacrylamide gels than the major phosphotyrosine-containing protein from RSV-transformed CEF. The fine-resolution analysis revealed the presence in the control cells of two phosphotyrosine peaks at approximately 40K and 42K M_r, which we have previously reported (32). EGF stimulation greatly increased the phosphotyrosine content of the larger protein (3.3-fold), with a smaller increase in the 40K M_r protein (1.6-fold). The major phosphotyrosine peak of the RSV-transformed CEF migrated considerably faster on the polyacrylamide gel, comigrating with the 35K M_r marker protein. When extracts of EGF-stimulat-

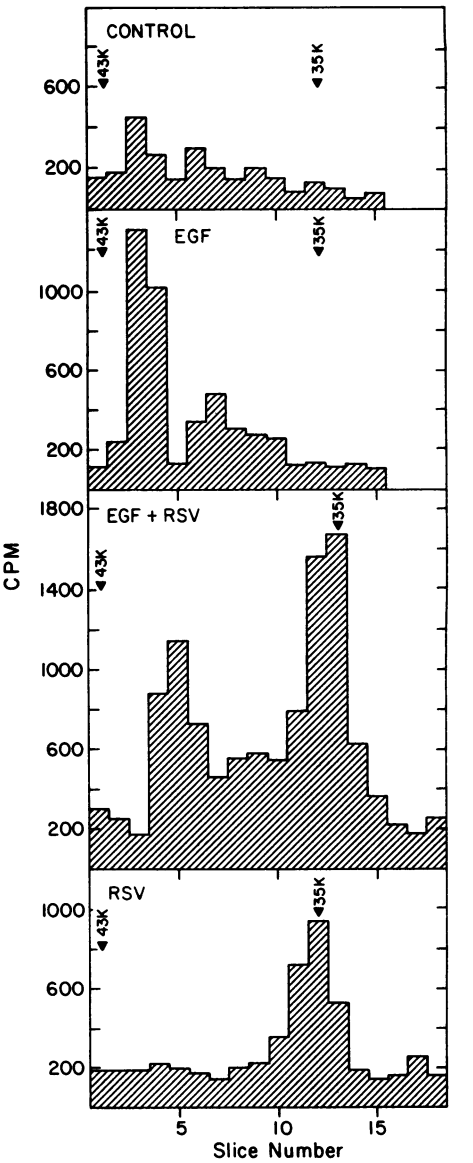

FIG. 7. Separation on polyacrylamide gels of the major phosphotyrosine-containing peaks of EGF-stimulated and RSV-transformed CEF. Both CEF and RSV-transformed CEF were labeled with $^{32}P_i$ for 6 to 8 h. The CEF were then treated for 5 min with EGF (500 ng/ml) before both cell types were lysed in hot Laemmli electrophoresis sample buffer and run on 10% polyacylamide tube gels. A portion of the gel below the 43K M_r dansylated marker (ovalbumin) and including the 35K M_r marker (lactate dehydrogenase) was cut into 1-mm slices, and the phosphotyrosine content of each slice was determined as described in the text. The third panel shows the results of running a mixed extract of EGF-stimulated and RSV-transformed CEF. The amounts of protein in the extracts used were not equal but were chosen to give approximately equal peak heights of the major phosphotyrosine peaks.

ed CEF and RSV-transformed CEF were mixed and run on the same gel, the major phosphotyrosine peaks were easily resolvable (Fig. 7).

EGF stimulation requires an EGF receptor. To determine whether the increased tyrosine phosphorylation we were observing was being mediated through unique receptors, we examined the effect of EGF on the phosphotyrosine profile of the NR-6 derivative of 3T3 cells, which lacks detectable EGF receptors (36), and on the parental 3T3 cells (Fig. 8). The similarity of the phosphotyrosine profiles of the unstimulated 3T3 and NR-6 cells gives a good indication of the reliability and reproducibility of our technique for quantitating phosphotyrosine changes. The addition of EGF to density-inhibited 3T3 cells resulted in a rapid tyrosine phosphorylation of the 40K to 42K M_r region of the gel in much the same manner as was seen with CEF. However, with the EGF-receptorless NR-6 cells, this increase in phosphotyrosine was hardly detectable.

DISCUSSION

We found that a rapid response of CEF to serum, EGF, PDGF, or MSA is an increased amount of tyrosine phosphorylation of proteins in the region of M_r 40K to 42K. Cooper and his collaborators, using a two-dimensional gel technique (14), have also found very similar increases in phosphotyrosine-containing proteins in 3T3 cells treated with EGF or PDGF (13a). The similarity of the induced changes in protein phosphorylation was surprising to us, since these mitogens are reported to bind to different receptors (5, 9, 11, 18, 25, 26, 39, 42). This suggests that the tyrosine phosphorylations we report here are somehow involved in the integration of the biological response to polypeptide mitogens.

It remains to be determined, however, whether these phosphorylations are involved in effecting the early biological responses to mitogens (such as increased hexose transport), the later responses (such as DNA synthesis), or neither. For example, recent results have shown that cyanogen bromide modification of EGF destroys the mitogenic potential of the protein without affecting its ability to induce phosphorylation of the EGF receptor or some early biological responses (43, 51). Thus, phosphorylation of the receptor is not sufficient to promote cell division, but may be sufficient to induce some early responses. It would be of some interest to determine whether phosphorylation of the 40K to 42K M_r proteins described here could be stimulated with EGF which had been modified with cyanogen bromide.

In the case of EGF stimulation of density-inhibited CEF, we have shown that the major increase in tyrosine phosphorylation takes place on a protein with M_r of 42K. A smaller increase in phosphorylation was seen for a 40K M_r pro-

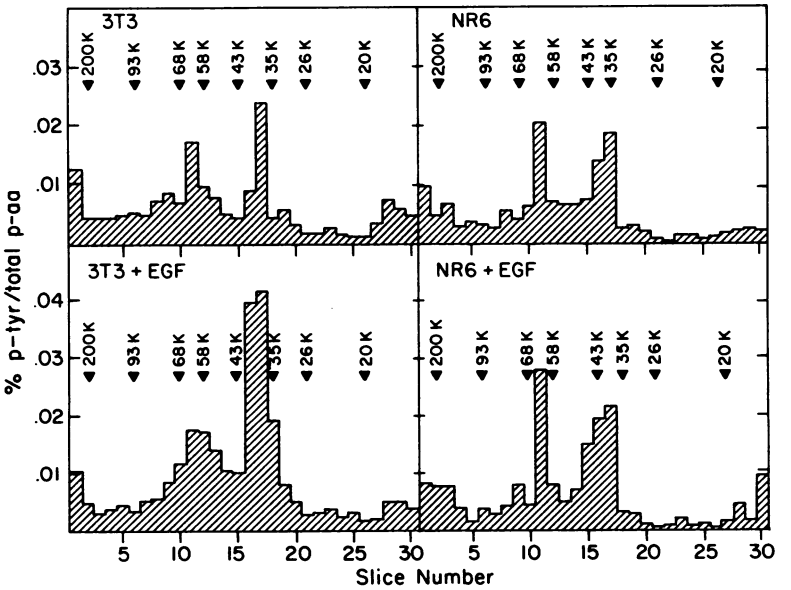

FIG. 8. Stimulation of phosphotyrosine (p-tyr) phosphorylation in 3T3 and NR-6 cells in response to EGF. Cells labeled with $^{32}P_i$ for 6 to 8 h were treated for 5 min with EGF (500 ng/ml). Phosphoamino acid (p-aa) analysis was carried out as described in the text. Only the results for phosphotyrosine are presented. Positions of dansylated molecular weight marker proteins are indicated by arrows.

tein. Both the 40K and 42K M_r peaks were found to be phosphorylated at a low level on tyrosine even in our unstimulated control cell cultures and are likely to be the same phosphoproteins that we reported earlier in sparse, growing CEF (32). Since serum is required for the growth of CEF in culture, it is possible that phosphorylation of these two proteins in the sparse, growing cultures was due to stimulation of cellular tyrosine kinases by growth factors in serum.

In the A431 human carcinoma cell line, the major tyrosine-phosphorylated protein detected after EGF stimulation is the same protein as the 36K protein which is phosphorylated in RSV-transformed cells (15, 19). However, we have not been able to detect any increase in phosphorylation of the 36K protein in either CEF or 3T3 cells after serum or growth factor stimulation. One possible explanation for this discrepancy may be related to the fact that EGF is not growth stimulatory for A431 cells but actually inhibits the growth of these cells (2, 24). In addition, A431 cells have very high levels of EGF receptors (8, 47). It may be that in situations where tyrosine kinase activity is exceptionally high (RSV-transformed cells and EGF-stimulated A431 cells), a large number of low-affinity, adventitious phosphorylations occur. Phosphorylation of the 36K protein may fall into this category. Strengthening this suggestion that A431 cells may not be an appropriate system to study the role of phosphotyrosine-containing proteins in mitogenesis is our observation (unpublished data) that in these cells an inordinately large number of proteins (at least 30) become phosphorylated on tyrosine after EGF treatment. This complexity is comparable to that observed with RSV-transformed cells (32). In any event, it is clear that phosphorylation of the 36K protein is not necessary for growth stimulation by mitogens. We previously had reached the conclusion that phosphorylation of the 36K protein was not necessary for the loss of density-dependent growth control induced by RSV, based on our analysis of the CU2 mutant of RSV, a *src* mutant which causes cells to lose their sensitivity to density-dependent growth control but does not cause 36K to become greatly phosphorylated (1, 34). Thus, two lines of evidence suggest that phosphorylation of the 36K protein is not involved in growth control. Phosphorylation of the 36K protein, if it is not adventitious, may be required for the appearance of some other manifestation of transformation, such as plasminogen activator (34).

There is evidence that the tyrosine kinase activity induced by EGF resides in the EGF receptor itself (8, 10, 11). There are specific PDGF and MSA receptors as well which are distinct from the EGF receptor (5, 18, 25, 26, 39). However, whether the tyrosine kinase activity induced by PDGF treatment also is associated with these receptors has not yet been determined, although the PDGF receptor can be phosphorylated on tyrosine (18). We have shown using the NR-6 cell line, a variant of the 3T3 line which lacks EGF receptors, that the tyrosine phosphorylation of the target proteins induced by EGF is mediated via the EGF receptor. When EGF was added to NR-6 cells, even at very high concentrations, there was very little stimulation of tyrosine phosphorylation of the 40K to 42K M_r proteins compared with the EGF stimulation of the parent 3T3 cells. However, it still is uncertain whether the 40K to 42K M_r proteins are primary targets of the receptor or are phosphorylated by some other kinase which is activated by the receptor.

The phosphorylations we observed were transitory and peaked within about 5 min. The decline in phosphorylation appears to be more rapid than the normal process of receptor degradation, but may be associated with receptor clustering and internalization (33, 50). However, it will require more detailed analyses to determine the kinetic and mechanistic relationship between these phosphorylations and receptor down-regulation.

On our protein profiles we have not detected phosphotyrosine-containing proteins with M_rs of around 160K to 170K, the approximate molecular weight of the receptors for EGF and PDGF reported for a variety of other cell types (5, 8, 11, 25). Nearly all of the increase in phosphotyrosine after mitogen stimulation has been found in the proteins of around 40K to 42K M_r. Thus, the phosphorylation of the receptor must constitute only a very small percentage of the total mitogen-induced tyrosine phosphorylation in these cells, if, indeed, it occurs at all.

An aim of this investigation was to identify proteins which become phosphorylated both in RSV-transformed cells and in response to mitogens. Proteins in the region of 40K to 42K M_r become phosphorylated on tyrosine in RSV-transformed cells, but because of the numerous phosphotyrosine-containing proteins in the 30K to 80K M_r region in the transformed cells, it is not possible to determine with our procedures whether the proteins described here are also phosphorylated in response to $pp60^{src}$. This determination will require investigations utilizing techniques with higher resolution, and such investigations are underway.

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