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Phosphorylation of Eukaryotic Translation Initiation Factor 4E Is Increased in Src-Transformed Cell Lines

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Eukaryotic initiation factor 4F (eIF-4F) is a three-subunit complex that binds the 5′ cap structure (m^7GpppX, where X is any nucleotide) of eukaryotic mRNAs. This factor facilitates ribosome binding by unwinding the secondary structure in the mRNA 5′ noncoding region. The limiting component of the 4F complex is believed to be the 24-kDa cap-binding phosphoprotein, eIF-4E. In this report, we describe the phosphorylation of eIF-4E in response to expression of the tyrosine kinase oncoproteins $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src527F}$. The results suggest that eIF-4E functions as a downstream target of the phosphorylation cascade induced by tyrosine-specific protein kinases as well as by effectors of the mitogenic response.

Treatment of cultured cells with growth-promoting agents results in an increase in protein synthesis, which is obligatory for quiescent cells to reenter the cell cycle (3). This increase in translation rates is believed to result from changes in the phosphorylation state of key translation factors (7). The overall rate-limiting step in translation initiation is the binding of eukaryotic mRNAs to ribosomes (9). This process is mediated by binding of the eukaryotic initiation factor 4F (eIF-4F) complex to the 5′ cap structure present on all eukaryotic cellular mRNAs (for reviews, see references 19 and 23). eIF-4F stimulates binding to ribosomes by unwinding the secondary structure in the mRNA 5′ noncoding region (18, 20). The 24-kDa cap-binding protein, eIF-4E (24), is the limiting component of the eIF-4F complex (4, 8) and thus a candidate key target for regulation of mRNA binding to ribosomes. Indeed, the cap-binding complex, eIF-4F, behaves as a discriminatory factor in in vitro translation systems (18, 22), and addition of eIF-4E to a HeLa translation system stimulates translation of capped mRNAs (25). Significantly, overexpression of eIF-4E in NIH 3T3 or Rat-2 fibroblasts has recently been shown to result in their malignant transformation (14). This result not only further documents the limiting nature of eIF-4E activity in the cell but also demonstrates that an initiation factor can be a proto-oncogene and indicates that regulation of gene expression at the translational level is critical for cell growth.

Evidence is mounting that one way of modulating the activity of this translation initiation factor in vivo is through its reversible phosphorylation. eIF-4E contains one major site of phosphorylation at Ser-53 (10, 21); decreased phosphorylation of eIF-4E has been correlated with the decrease in protein synthesis during heat shock (4) and mitosis (2). Enhanced phosphorylation of eIF-4E has been observed in 3T3-L1 cells stimulated with phorbol esters and insulin (16, 17) as well as in human cervical carcinoma cells treated with tumor necrosis factor α (15) and serum-stimulated Swiss 3T3 cells (11). Significantly, mutation of Ser-53 to alanine prevents the association of eIF-4E with the 48S initiation complex (10). Taken together, these results suggest that regulated phosphorylation of eIF-4E plays a role in the alteration of translation efficiency in response to growth-modulating conditions. In this report, we demonstrate enhanced eIF-4E phosphorylation in cells expressing transforming variants of the tyrosine-specific protein kinase $pp60^{src}$.

eIF-4E immunoprecipitation. eIF-4E was immunoprecipitated from ^{32}P-labeled cultured cells with a rabbit anti-eIF-4E polyclonal antiserum. This antiserum was raised against a synthetic murine eIF-4E peptide containing amino acids 5 to 23. Figure 1 shows a polyacrylamide gel electrophoresis (PAGE) analysis of eIF-4E purified by immunoprecipitation (lanes 5 to 7) compared with m^7GDP affinity chromatography (lanes 1 to 4). For this and all subsequent experiments, subconfluent monolayers of cells on 60-mm plates were metabolically labeled by incubation with 1.5 ml of $^{32}P_i$ (0.33 mCi/ml; NEN) in phosphate-free Dulbecco modified essential medium (DMEM; Flow Laboratories) for the indicated times (2 h for this experiment). Monolayers were subsequently rinsed in cold (4°C) 1× phosphate-buffered saline (PBS) and lysed for 15 min in 1 ml of ice-cold RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium desoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5]; for immunoprecipitation) plus phosphatase inhibitors (50 mM NaF, 2 mM $NaPP_i$, 1 mM sodium orthovanadate, 80 mM β-glycerophosphate, 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride). The lysate was transferred to a 1.5-ml Eppendorf tube and clarified by centrifugation. Protein was estimated by the Pierce assay (Pierce Corp.), and aliquots of lysate containing equivalent amounts of total protein were used for the immunoprecipitation experiments, in a total volume of 750 μl. Cell lysates were incubated with 2 to 5 μl of antiserum overnight on ice; 100 μl of 10% protein A-Sepharose CL-4B (Pharmacia) in 1× PBS was added, and incubation continued for 1 h on a rotary wheel at 4°C. Immunocomplexes were collected with 100 μl of sample buffer and electrophoresed (50 μl) on a 0.1% SDS–12.5% polyacrylamide gel (SDS-PAG). The immune serum (Fig. 1, lane 6), but not the preimmune serum (lane 5), specifically precipitated a phosphoprotein with a mobility indistinguishable from that of eIF-4E purified on m^7GDP-agarose (lane 4); preabsorption of the antiserum with the synthetic eIF-4E peptide blocked this interaction (lane 7).

Reduced phosphorylation of eIF-4E upon serum starvation. Mammalian fibroblast cell lines, such as 3T3, are rendered quiescent upon deprivation of serum and exhibit a very slow

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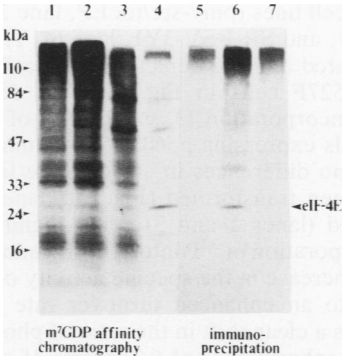

FIG. 1. eIF-4E purification by m^{7}GDP affinity chromatography and immunoprecipitation. Material from one plate of ^{32}P-labeled NIH 3T3 cells (as described in the text), lysed in 1 ml of lysis buffer (14) containing phosphatase inhibitors (see text), was subjected to purification on m^{7}GDP-agarose as described previously (6) and electrophoresed on a 12.5% SDS-PAG. Lane 1, Load (3 μl of 1 ml); lane 2, flowthrough (3 μl); lane 3, first GDP eluate (300 μl); lane 4, first m^{7}GDP eluate (300 μl). Immunoprecipitation was performed as described in the text with either preimmune (lane 5) or immune (lane 6) serum or immune serum preabsorbed with 100 μg of the antigenic peptide (lane 7). One-half of the immunoprecipitated material was electrophoresed with the m^{7}GDP-agarose column fractions on a 12.5% SDS-PAG. Protein standards are Bio-Rad low-range prestained markers.

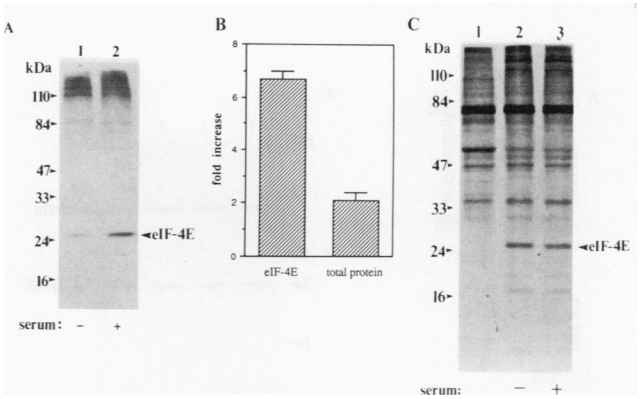

FIG. 2. Effect of serum on ^{32}P incorporation into eIF-4E. (A) SDS-PAGE analysis of immunoprecipitated eIF-4E from ^{32}P-labeled cells. Plates were seeded with 10^6 NIH 3T3 cells and allowed to grow overnight in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in a 37°C, 5% CO_2 humidified environment. The next day, cells were serum deprived by incubation in serum-free DMEM for 4 h and then equilibrated with $^{32}P_i$ (0.33 mCi/ml; NEN) in phosphate-free DMEM for 90 min. Subsequently, 10% dialyzed fetal bovine serum (GIBCO) was added to one set of serum-deprived cells and labeling was allowed to continue for another hour. Cells were then lysed, and eIF-4E was immunoprecipitated as described in the text. Lanes: 1, serum-deprived cells; 2, serum-stimulated cells. (B) Quantitative analysis of eIF-4E phosphorylation. Autoradiographs of phosphorylated eIF-4E were scanned densitometrically for three separate experiments. Fold increase represents levels of $^{32}P_i$ incorporation in serum-stimulated cells divided by levels in serum-deprived cells. Incorporation of ^{32}P radiolabel into total protein was measured by scintillation counting of trichloroacetic acid-precipitable material. (C) Immunoprecipitation and SDS-PAGE analysis of eIF-4E metabolically labeled with [^{35}S]methionine. Cells were plated and serum deprived as described for the $^{32}P_i$ labeling experiments. Labeling of cells was done with 1.5 ml of 0.67-mCi/ml [^{35}S]methionine for 2.5 h. Lanes: 1, immunoprecipitation with preimmune serum; 2, serum-deprived cells immunoprecipitated with immune serum; 3, cells stimulated with 10% dialyzed fetal bovine serum for 1 h prior to lysis and immunoprecipitation of eIF-4E.

rate of entry into S phase; this effect is reversed upon readdition of serum (3). Using m^{7}GTP affinity chromatography, Kaspar et al. showed an increased rate of incorporation of $^{32}P_i$ into eIF-4E upon treatment of Swiss 3T3 cells with serum (11). To determine whether immunoprecipitation is an effective method to detect changes in eIF-4E phosphorylation, we examined the extent of eIF-4E phosphorylation in quiescent and serum-stimulated NIH 3T3 cells. Cells were metabolically labeled with $^{32}P_i$, and eIF-4E was immunoprecipitated and subjected to PAG electrophoresis (PAGE). A representative result demonstrates increased incorporation of $^{32}P_i$ into eIF-4E in serum-stimulated cells (Fig. 2A, lane 2). This effect was quantitated and related to changes in incorporation of the radiolabel into total cellular protein (Fig. 2B); cells that were restimulated by addition of 10% serum 1 h prior to lysis and immunoprecipitation showed a specific increase (3.4 ± 0.4-fold) in incorporation of $^{32}P_i$ into eIF-4E. To show that this increase does not reflect a general increase in $^{32}P_i$ incorporation due to changes in the levels of eIF-4E protein upon serum stimulation, eIF-4E was immunoprecipitated from cells which had been metabolically labeled with [^{35}S]methionine; no difference in the level of eIF-4E protein synthesis was detected under these conditions (Fig. 2C; compare lanes 2 and 3). Furthermore, cell lysates immunoblotted with the anti-eIF-4E antiserum and a monoclonal antiactin antibody revealed no changes in steady-state eIF-4E protein levels between quiescent and serum-stimulated 3T3 cells (Fig. 3C; compare lanes 1 and 3). Thus, both the rate of synthesis and the steady-state level of eIF-4E are unaffected by serum stimulation, indicating that eIF-4E protein levels are not regulated by serum. We conclude that immunoprecipitation is a quick and effective means of detecting differences in eIF-4E phosphorylation, as our results are similar to previously published reports of fold stimulation of phosphorylation of eIF-4E upon serum stimulation (11). Thus, this method can be used to examine the extent of eIF-4E phosphorylation in cells transformed by tyrosine protein kinase oncogenes.

Expression of transforming variants of pp60^{src} enhances eIF-4E phosphorylation in serum-starved 3T3 cells. 3T3 cells transformed by viral tyrosine kinase oncoproteins, such as pp60$^{v\text{-}src}$, continue to grow and divide upon serum deprivation, in contrast to nontransformed parental lines. This characteristic is believed to be due to the ability of tyrosine kinase oncoproteins to stimulate the phosphorylation, and thus presumably the activity, of putative key cellular targets in the mitogen-activated signaling cascade. Figure 3A shows an SDS-PAGE analysis of phosphorylated eIF-4E, immunoprecipitated from nontransformed parental cell lines (murine NIH 3T3 and rat 3Y1) and previously characterized derivative lines (5, 12) which overexpress either cellular pp60$^{c\text{-}src}$ (pmc-src/focB) or transforming Src derivatives, including viral pp60$^{v\text{-}src}$ (pmv-src/focEP and SR-3Y1) and pp60$^{c\text{-}src527F}$ (pmc-src527F/focEP). pp60$^{c\text{-}src527F}$ is a cellular Src protein which has been rendered transforming by substitution of the tyrosine at amino acid position 527 with phenylalanine (12). Whereas the levels of incorporation of the ^{32}P radiolabel into eIF-4E are characteristically low in the untransformed cell lines (3T3, lane 1; pmc-src/focB, lane 3; and 3Y1, lane 5) upon serum deprivation, ^{32}P incorporation is increased in the

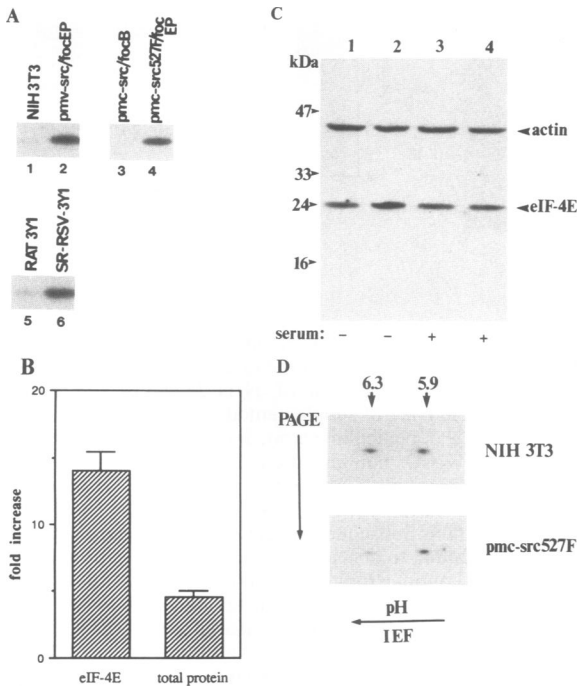

FIG. 3. Induction of eIF-4E phosphorylation by pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src527F}$. (A) Immunoprecipitation and SDS-PAGE analysis of eIF-4E from ^{32}P-labeled cells. Cell lines pmv-src/focEP, pmc-src527F/focEP, and pmc-src/focB are focus-selected clones from NIH 3T3 cells transfected with plasmids encoding pp60$^{v\text{-}src}$, pp60$^{c\text{-}src527F}$, and pp60$^{c\text{-}src}$, respectively (12). SR-3Y1 cells are derived from the immortalized, untransformed rat 3Y1 cell line by transformation with a wild-type Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) (5). Cells were plated, serum deprived, ^{32}P labeled (2 h), and processed as in previous experiments. Shown are representative results comparing eIF-4E immunoprecipitated from pmv-src/focEP (lane 2, expressing viral pp60$^{v\text{-}src}$), pmc-src527F/focEP (lane 4, expressing the transforming cellular pp60$^{c\text{-}src527F}$), and SR-RSV-3Y1 (lane 6, expressing viral pp60$^{v\text{-}src}$) and the respective parental (or control) lines: NIH 3T3 (lane 1), pmc-src/focB (lane 3, expressing the normal cellular pp60$^{c\text{-}src}$), and rat 3Y1 (lane 5). (B) Quantitative analysis of specific increase in eIF-4E phosphorylation in Src-expressing cells. Analysis was performed as described in the legend to Fig. 2. Levels of phosphorylation of both eIF-4E and total protein were compared in NIH 3T3 and pmc-src527F cells in three separate experiments. (C) Immunoblot analysis of eIF-4E and actin levels in NIH 3T3 and pmc-src527F cells as described by Lazaris-Karatzas et al. (14). A representative analysis is shown. Lanes: 1 and 3, NIH 3T3; 2 and 4, pmc-src527F; 1 and 2, serum-deprived cells; 3 and 4, serum-stimulated cells. (D) Two-dimensional IEF of eIF-4E in NIH 3T3 and pmc-src527F cells. Cells were plated and deprived of serum as described in the legend to Fig. 2; cells were then washed with 1× PBS, lysed in 400 μl of urea lysis buffer (9.5 M urea, 2% Nonidet P-40, 1.6% pH 5 to 7 ampholines, 0.4% pH 3 to 10 ampholines, 5% β-mercaptoethanol), loaded (50 μl) immediately on IEF tube gels (Bio-Rad), and focused overnight. Tube gels were electrophoresed in the second dimension on 12.5% SDS-PAGs and transferred to nitrocellulose for immunoblotting with eIF-4E serum and ^{125}I-protein A (Amersham) as described previously (14). Phosphorylated and unphosphorylated forms of eIF-4E are indicated by their pIs (5.9 and 6.3, respectively). Protein was measured in duplicate plates by the Pierce assay; 50% more protein was used for 3T3 cells.

transformed cell lines (pmv-src/focEP, lane 2; pmc-src527F/focEP, lane 4; and SR-RSV-3Y1, lane 6). These differences were quantitated and are depicted graphically for NIH 3T3 and pmc-src527F cells in Fig. 3B. We detect a specific increase of incorporation (3 ± 0.6-fold) of radiolabel into eIF-4E in cells expressing pp60$^{c\text{-}src527F}$. Immunoblot analysis revealed no differences in steady-state levels of eIF-4E protein between transformed (Fig. 3C, lanes 2 and 4) and untransformed (lanes 1 and 3) cells. Significantly, the increased incorporation of ^{32}P into eIF-4E cannot be attributed solely to an increase in the specific activity of the phosphate moiety due to an enhanced turnover rate in transformed cells. There is a clear shift in the ratio of phosphorylated (pI 5.9) to unphosphorylated (pI 6.3) forms of eIF-4E between NIH 3T3 cells (phosphorylated/unphosphorylated, 1:1) and cells expressing pp60$^{c\text{-}src527F}$ (3:1), as determined by two-dimensional isoelectric focusing (IEF) analysis of cell lysates (Fig. 3D). Nevertheless, some contribution of enhanced turnover of the phosphate in Src-expressing cells cannot be ruled out.

Enhanced eIF-4E phosphorylation in response to pp60^{src} expression occurs solely at the major site of serine phosphorylation. Detection of the two previously described isoelectric variants of eIF-4E (21) by two-dimensional IEF analysis (Fig. 3D) is consistent with the notion that the increased phosphorylation of eIF-4E in response to Src expression occurs at the previously described single major site of eIF-4E phosphorylation, Ser-53. However, we wished to rule out the possibility of minor secondary sites, possibly on tyrosine. To this end, phosphoamino acid and phosphopeptide analyses were performed on eIF-4E isolated from both phorbol-12-myristate-13-acetate (PMA)-stimulated NIH 3T3 cells and the pp60$^{v\text{-}src}$-expressing cells, pmv-src/focEP. eIF-4E phosphorylation in NIH 3T3 control cells (Fig. 4A) and in PMA-treated (Fig. 4B) or pp60$^{v\text{-}src}$-transformed (Fig. 4C) 3T3 cells occurred only on serine, indicating that Src-induced enhancement of eIF-4E phosphorylation is indirect. Longer exposures reveal no trace of phosphotyrosine or phosphothreonine (data not shown). Indeed, only a single tryptic phosphopeptide (Fig. 4D; presumably phosphoseryllysine, according to references 10 and 21) was detected after PMA treatment (Fig. 4E) or pp60$^{v\text{-}src}$ expression (Fig. 4F). Furthermore, mixing of these phosphopeptides prior to electrophoresis and chromatography (Fig. 4G and H) verified their identity. These results suggest that the same or similar factors are responsible for the enhancement of eIF-4E phosphorylation detected upon either PMA treatment or Src expression.

Induction of eIF-4E phosphorylation is an early event following treatment of 3T3 cells with serum, PMA, or PDGF. The results presented in this report provide evidence that the extent of eIF-4E phosphorylation correlates with the modulation of cell growth by a tyrosine protein kinase oncoprotein. One intriguing possibility is that eIF-4E is a necessary, albeit not sufficient, early effector of the mitogenic response triggered by tyrosine protein kinases as well as diverse mitogenic agents (11, 15–17). To demonstrate that phosphorylation of eIF-4E is an early response to extracellular mitogenic stimuli, we wished to compare the time course of the change in phosphorylation status of eIF-4E in response to different mitogens and growth factors. Increases in eIF-4E phosphorylation were detectable within 5 to 10 min after treatment with serum, the tumor promoter and mitogen PMA, and platelet-derived growth factor (PDGF), reaching a maximum at 30 min for PDGF and 2 h for PMA and serum (Fig. 5); no increases in phosphorylation were seen in

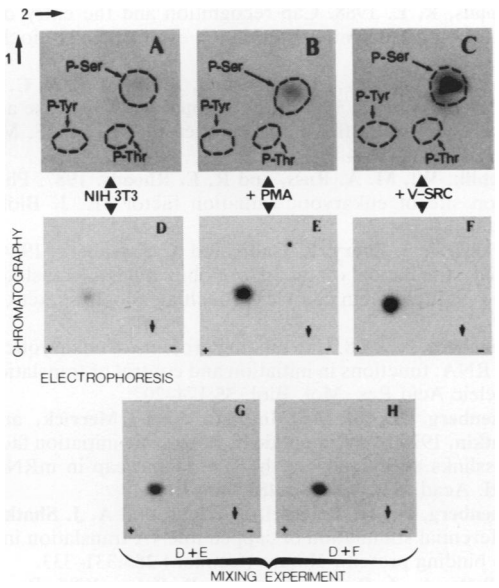

FIG. 4. Two-dimensional phosphoamino acid and phosphopeptide analyses. Analyses were performed on eIF-4E from ^{32}P-labeled serum-deprived NIH 3T3 cells. Cells were plated, ^{32}P labeled, and immunoprecipitated as described in the legend to Fig. 2. Following immunoprecipitation and 12.5% PAGE, eIF-4E was excised and eluted from the dried gel and subjected to acid hydrolysis or trypsin digestion for the phosphoamino acid (26) or phosphopeptide (27) analyses, respectively, as previously described. Phosphoamino acids (A to C) and phosphopeptides (D to H) were detected by autoradiography. (A and D) Untreated NIH 3T3 cells; (B and E) PMA-treated 3T3 cells; (C and F) pp60$^{v\text{-}src}$-expressing 3T3 cells (pmv-src/focEP in Fig. 2B); (G and H) mixing of eIF-4E phosphopeptides from PMA-treated 3T3 cells (G) or from cells expressing pp60$^{v\text{-}src}$ (H) with equivalent counts of peptides from untreated serum-deprived NIH 3T3.

mock-treated cells (data not shown). Thus, enhanced eIF-4E phosphorylation in ^{32}P-labeled 3T3 cells is a relatively early event in the mitogenic signaling cascade.

Our results suggest that transforming Src variants converge upon the regular signal transduction pathways which control eIF-4E phosphorylation, releasing cells from the requirement for extracellular signals to modulate this phosphorylation; this effect correlates with the ability of these cells to continue to grow and divide in the absence of serum in the growth media. Enhanced incorporation of the ^{32}P radiolabel into eIF-4E in Src-expressing cells is not accounted for merely by increased turnover; we see a clear shift toward the phosphorylated isoelectric variant in these cells. This effect is not restricted to Src, as increased phosphorylation of eIF-4E has also been detected in response to expression of transforming variants of Lck, a lymphocyte-specific tyrosine protein kinase (6a), and in cells expressing v-*fps* (6b).

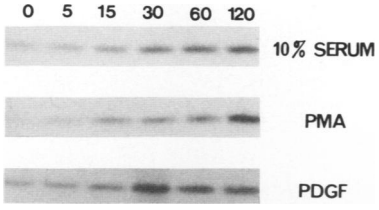

FIG. 5. Time course of eIF-4E phosphorylation upon treatment of 3T3 cells with serum, PMA, and PDGF. NIH 3T3 cells were plated, serum deprived, and ^{32}P labeled as described for Fig. 2. Dialyzed fetal calf serum (10%), PMA (100 ng/ml; Pharmacia), or PDGF (15 ng/ml; Collaborative Research) was added, and labeling continued for the indicated times (in minutes). Cells were lysed and lysates were processed as described in the text.

Enhanced eIF-4E phosphorylation in cells expressing the Src tyrosine kinase oncoprotein could be explained through direct or indirect activation of an eIF-4E kinase (and possibly an eIF-4E phosphatase, as mentioned earlier). A precedent for such an interaction exists for the family of S6 kinases, which are activated under a set of conditions similar to those that have been shown to induce eIF-4E phosphorylation (13). More significantly, S6 kinases are activated, most probably indirectly, in response to expression of transforming Src proteins (1). The results presented here, taken together with the recent demonstration of the transforming capacity of overexpressed eIF-4E (14), lead us to suggest an important role for eIF-4E phosphorylation in the mitogenic response. However, direct proof for such a role must await the isolation and characterization of cellular proteins capable of regulating this phosphorylation and the development of means of interfering with the action of such enzymes in vivo.

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