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Phosphorylation of eIF-4E and Initiation of Protein Synthesis in P19 Embryonal Carcinoma Cells

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Abstract Mitogenic stimulation of protein synthesis is accompanied by an increase in eIF-4E phosphorylation. The effect on protein synthesis by induction of differentiation is less well known. We treated P19 embryonal carcinoma cells with the differentiating agent retinoic acid and found that protein synthesis increased during the first hour of addition. However, the phosphorylation state, as well as the turnover of phosphate on eIF-4E, remained unchanged. Apparently, the change in protein synthesis after RA addition is regulated by another mechanism than eIF-4E phosphorylation.

By using P19 cells overexpressing the EGF receptor, we show that the signal transduction pathway that leads to phosphorylation of eIF-4E is present in P19 cells; the EGF-induced change in phosphorylation of eIF-4E in these cells is likely to be regulated by a change in eIF-4E phosphatase activity.

These results suggest that the onset of retinoic acid-induced differentiation is triggered by a signal transduction pathway which involves changes in protein synthesis, but not eIF-4E phosphorylation.

Key words: protein synthesis, retinoic acid, EGF, NGF, differentiation, phosphatase, eIF-4E

Eukaryotic translation initiation is regulated by phosphorylation of initiation factors. It has been shown that changes in the phosphorylation state of four of these eukaryotic initiation factors (eIF), eIF-2, eIF-2B, eIF-4B, and eIF-4E, coincide with changes in protein synthesis [Redpath and Proud, 1994]. The first two proteins act to deliver Met-tRNA onto the 40S ribosomal subunit: eIF-2 by complexing to Met-tRNA and GTP, and eIF-2B by replacing GDP on eIF-2 for GTP. Phosphorylation of the α-subunit of eIF-2 impaired the GDP-GTP exchange by sequestration of eIF-2B [Konieczny and Safer, 1983; Salimans et al., 1984; Siekierka et al., 1982]. In vitro experiments showed that phosphorylation of the ϵ-subunit of eIF-2B increased the GDP/GTP exchange activity, enabling a higher protein synthesis rate [Dholakia and Wahba, 1988; Singh et al., 1994; Welsh and Proud, 1992].

eIF-4E binds the cap-structure on the mRNA [Sonenberg et al., 1978; Rhoads, 1988]. Subsequent unwinding of the secondary structure of the 5′untranslated region (UTR) [Rozen et al., 1990] enables binding of the 40S · eIF-2 · Met-tRNA · GTP complex followed by formation of the 80S ribosomal complex.

eIF-4A, together with eIF-4B, is able to unwind double-stranded RNA or RNA/DNA duplexes [Lawson et al., 1989; Rozen et al., 1990]. eIF-4B can be multiply phosphorylated in response to mitogens [Duncan and Hershey, 1985; Wolthuis et al., 1993]. A decrease in phosphorylation of eIF-4B is observed after serum starvation of cells. The role of phosphorylation of eIF-4B on the protein synthesis rate remains to be determined.

Phosphorylation of the cap-binding protein eIF-4E closely follows the protein synthetic rate. Phosphorylation of eIF-4E is decreased after adenovirus infection [Huang and Schneider, 1991], heat shock [Lamphear and Panniers, 1991], and mitosis [Bonneau and Sonenberg, 1987]. In all cases a decrease in protein synthesis was detected. An increase in eIF-4E phosphorylation was shown after treatment of cells with mitogens, like phorbol esters [Frederickson et al., 1991; Morley and Traugh, 1989; Rychlik et al., 1990], and lipopolysaccharide (LPS) [Rychlik et al., 1990], treatment with insulin [Manzella et al., 1991; Morley and Traugh, 1990], serum [Kaspar et al., 1990], epidermal growth

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factor (EGF) [Donaldson et al., 1991], tumor necrosis factor (TNF) [Marino et al., 1991], platelet-derived growth factor (PDGF) [Bu and Hagedorn, 1991; Frederickson et al., 1991], nerve growth factor (NGF) [Frederickson et al., 1992], and overexpression of $pp60^{src}$ [Frederickson et al., 1991] or $p21^{ras}$ [Rinker-Schaeffer et al., 1992]. After treatment of cells with phorbol esters, serum, and after $p21^{ras}$ transformation an increase in protein synthesis was detected. Thus, the numerous examples indicate that mitogenic stimulation of cells leads to an increased phosphorylation status of elF-4E and an increased protein synthesis.

Phosphorylation of elF-4E seems to be necessary for efficient translation initiation. First, 80 to 85% of elF-4E present on the 48S initiation complex was phosphorylated [Joshi-Barve et al., 1990; Lamphear and Panniers, 1990]; in contrast, only 50% of the free elF-4E was phosphorylated. Second, phosphorylated elF-4E binds m^7GTP, m^7GpppG, and globin mRNA 3 to 4 times more efficiently than nonphosphorylated elF-4E [Minich et al., 1994].

An increase in elF-4E phosphorylation is believed to enhance translation of mRNAs with an extensive secondary structure in their 5′UTR by an increase in the activity of elF-4E. The ornithine decarboxylase G/C-rich 5′UTR was more efficiently translated when cells were stimulated with insulin or phorbol ester [Manzella et al., 1991]. Furthermore, mRNAs in which secondary structure was increased by cloning complementary regions in the 5′UTR were poorly translated in normal cells, but translation was relatively efficient in cells overexpressing elF-4E [Koromilas et al., 1992].

The amount of elF-4E present in the cell limits the initiation process [Duncan et al., 1987; Hiremath et al., 1985]. It is expected that mRNAs with extensive folding are only translated under conditions when more phosphorylated elF-4E is present. Proteins such as oncogene products, growth factors, and growth factor receptors are often encoded by mRNAs with a complex secondary structure in the 5′UTR. These proteins play a critical role in growth, differentiation, and development [Kozak, 1987; Lazaris-Karatzas et al., 1992].

Pheochromocytoma (PC12) cells can be differentiated with NGF. Treatment of PC12 cells with NGF led to a rapid increase in elF-4E phosphorylation, suggesting that elF-4E phosphorylation plays a role in the change of gene expression at the very early onset of differentiation [Frederickson et al., 1992]. Inhibition of ras-activity in PC12 cells by a dominant negative mutant led to a decreased elF-4E phosphorylation upon NGF addition. It was also found that ras-inhibition led to a marked repression of the neurite outgrowth of PC12 cells after NGF treatment. These two findings suggest that elF-4E phosphorylation and ras-activation are necessary for differentiation of PC12 cells [Frederickson et al., 1992]. The question can be raised whether phosphorylation of elF-4E is obligatory for differentiation in general. The amount of phosphorylated elF-4E may be able to regulate cell growth and differentiation: overexpression of elF-4E in HeLa cells [de Benedetti and Rhoads, 1990], NIH 3T3, and rat 2 fibroblasts [Lazaris-Karatzas et al., 1990] led to aberrant growth of the cells. Stimulation of translation initiation of mRNAs with a complex 5′UTR by phosphorylation of elF-4E suggests that elF-4E may be involved in the regulation of differentiation [Lazaris-Karatzas et al., 1992]. To determine whether differentiation coincides with a change in elF-4E phosphorylation, P19 embryonal carcinoma cells were treated with the differentiation-inducing agent retinoic acid (RA). P19 cells are malignant stem cells of teratocarcinomas. Depending on the chemical agent (retinoic acid [RA] or dimethyl sulfoxide [DMSO]) and the culture conditions used [Jones-Villeneuve et al., 1982; McBurney et al., 1982], P19 cells can differentiate into cells representing the three germ layers of the mouse embryo: endoderm, ectoderm, and mesoderm [Martin, 1980]. In this respect, P19 cells are very similar to the pluripotent inner cell mass of early embryos. P19 cells which overexpress a human EGF receptor (EGF-R) were treated with EGF to determine whether the signal transduction pathway leading to elF-4E phosphorylation is present in undifferentiated P19 cells.

MATERIALS AND METHODS

Materials

Acrylamide/bisacrylamide and biolytes were from Biorad, Veenendaal, the Netherlands; CHAPS from Boehringer, Almere, the Netherlands; urea and G418 from Gibco/BRL, Breta, the Netherlands; all-trans retinoic acid and 7S-NGF from Sigma, Brunschwigchemie, Amsterdam, the Netherlands; and murine EGF from Harlan, CPB, Zeist, the Netherlands.

Cell Culture

P19 cells were cultured in a 1:1 mixture of Ham F-12 and Dulbecco modified Eagle's medium (DF) supplemented with 7.5% fetal calf serum. P19-8-39 cells were obtained by cotransfection of pSV2neo and pSV2HERc, an expression vector for the human EGF-R [den Hertog et al., 1991a]. P19-8-39 cells were cultured as P19 cells, but in the presence of G418 (200 μg/ml). P19-8-39 cells will be referred to as P19-EGF cells.

Treatment of Cells With Retinoic Acid or EGF and Measurement of the Protein Synthesis

P19 cells and P19-EGF cells were grown in 6-cm-diameter tissue culture dishes to a confluency of 70%. The cells were deprived of serum in the presence of 10 μg transferrin per ml for the times indicated in the figure legends before treatment with RA (10^{-6} M) or EGF (50 ng/ml). Cells were washed twice with phosphate-buffered saline (PBS) and harvested in sample buffer for one-dimensional (1D) iso-electric focusing, containing 9.5 M urea, 80 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.75% Biolytes 3/10, 2.25% Biolytes 4/6, and 700 mM β-mercaptoethanol. Before harvesting, the cells were labeled with 12.5 μCi of [^{35}S]methionine/cysteine (Amersham, Den Bosch, the Netherlands) per ml for the times indicated in the figure legends. Incorporation of [^{35}S]methionine/cysteine into protein was measured by trichloroacetic acid precipitation.

Detection of the Phosphorylation State of eIF-4E by 1D-IEF

Cell lysates were run on a denaturing 1D iso-electric focusing gel (6% acrylamide/bisacrylamide (19:1), 9 M urea, 30 mM CHAPS, 0.75% Biolyte 3/10, 2.25% Biolyte 4/6) as described [Maurides et al., 1989]. The system was modified for a 7 × 8 cm-gel system. Electrophoresis time was 3.5 h. The gel was blotted onto a PVDF membrane using a semidry apparatus (Hoefer, San Francisco, CA). eIF-4E was detected by using a purified polyclonal antibody raised against a eIF-4E peptide (EPETTPTTNPPPAEEEKT) [Jaramillo et al., 1991]. Antibodies were purified by binding to eIF-4E-peptide coupled to Sepharose. Bound antibodies were eluted with 0.1 M $NaHCO_3$, pH 11.5, and immediately neutralized with 1 M HAc. The blot was developed with the alkaline phosphatase method and the percentage of phosphorylated eIF-4E over the total amount of eIF-4E was determined with a densitometer (Molecular Dynamics).

Treatment of PC12 Cells With NGF

PC12 cells were grown in DF medium containing 7.5% fetal calf serum in 6-cm-diameter tissue culture dishes to a confluency of 50%. The cells were deprived of serum for 2 h, before treatment with NGF (30 ng/ml). Cell lysates were prepared, run on a 1D iso-electric focusing gel, and analyzed as described above.

Incorporation of [^{32}P]-Labeled Orthophosphate Into eIF-4E

P19 cells and P19-EGF cells were grown to a confluency of 70%, and deprived of serum as described above. Cells were washed with phosphate-free DF and incubated in the same medium containing 100 μCi of carrier-free [^{32}P]-labeled orthophosphate (Amersham) per ml. Cells were labeled for the times indicated in Figure 3 up to 3 h, to determine the optimal labeling conditions for eIF-4E. After washing with PBS, the cells were lysed with 400 μl 10 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, adjusted with KOH, 50 mM β-glycerophosphate, 0.2 mM EDTA, 0.5% Nonidet-P40, 100 mM KCl, 7 mM β-mercaptoethanol, 0.2 mM Na_3VO_4, 0.2 mM benzamidine, and 4 μg leupeptin per ml. The lysates were centrifuged at 10,000*g* to remove cell debris and the supernatant was used to purify eIF-4E. The supernant was incubated with 5 μl packed m^7GTP Sepharose, supplemented with 15 μl packed Sepharose 4B to increase the pellet volume. After 1 h the beads were washed 3 times with lysis buffer and eIF-4E was eluted with 10 mM HEPES-KOH, pH 7.5, 0.2 mM EDTA, 100 mM KAc, and 75 μM m^7GTP. The eluant was analyzed by sodium dodecyl sulphate (13.5%) polyacrylamide gelelectrophoresis (SDS-PAGE) and phosphorylation of eIF-4E was quantified with a phospho-imager (Molecular Dynamics).

Turnover of Phosphorylated 4E Measured by [^{32}P]orthophosphate Labeling

P19 and P19-EGF cells were grown and labeled as described above. After 3 h of labeling, medium was replaced by DF with or without RA or EGF. Cells were harvested at the times indicated in the legend. eIF-4E was purified with

m^{7}GTP Sepharose and analyzed by SDS-PAGE (13.5%) as described above.

RESULTS

Protein Synthesis and the eIF-4E Phosphorylation State in P19 Cells

Serum deprivation of P19 cells for 1.5 h caused a decrease in protein synthesis to 90% (Table I). After 24 h of serum starvation, protein synthesis was 23% of that of the control cells. The phosphorylation state of eIF-4E was measured at the same time points by using 1D iso-electric focusing. During the first 24 h of serum starvation no change was detected in the eIF-4E phosphorylation state, in spite of the change in protein synthesis. Only after 48 h a decrease in the eIF-4E phosphorylation state was detected from 30 to 12%. Apparently, no correlation exists between protein synthesis and the eIF-4E phosphorylation state after serum deprivation of P19 cells. After the indicated periods of serum starvation, the cells were treated with RA or serum for 30 min. After 1.5 h of serum deprivation both parameters were unchanged. After 16 to 40 h of starvation, both RA and serum treatment caused a small but reproducible increase in protein synthesis. However, the phosphorylation state of eIF-4E remained at the same level. No correlation was found between protein synthesis and the eIF-4E phosphorylation state in P19 cells during serum starvation or after treatment with RA or serum.

Table I shows the results of treatment of P19 cells with RA at one time point of induction only (30 min). To determine whether eIF-4E phosphorylation occurred transiently as also found in PC12 cells [Frederickson et al., 1992], P19 cells were treated with RA for up to 4 h (Fig. 1). The control lane (Fig. 1, lane 3) showed that 31% of eIF-4E was phosphorylated after serum deprivation for 24 h. The phosphorylation state after serum starvation was the same as found for nontreated cells (Fig. 1, lane 1). Treatment with RA (lanes 4–10) did not lead to a significant change in the phosphorylation pattern. However, protein synthesis increased 50% in the first hour, as also shown in Table I, and decreased again after 90 min. Stimulation of serum-deprived cells with serum led to an increase in protein synthesis, but no significant change in the phosphorylation state was detected (Fig. 1, lane 2).

The phosphorylation state of eIF-4E was also followed during 3 days of differentiation after RA addition. No changes were detected, although protein synthesis gradually declined from 100 to 30%. This decrease in protein synthesis coincided with a decrease in cell growth and DNA synthesis. Together with the change in morphology, this showed that RA treatment caused differentiation of P19 cells (data not shown).

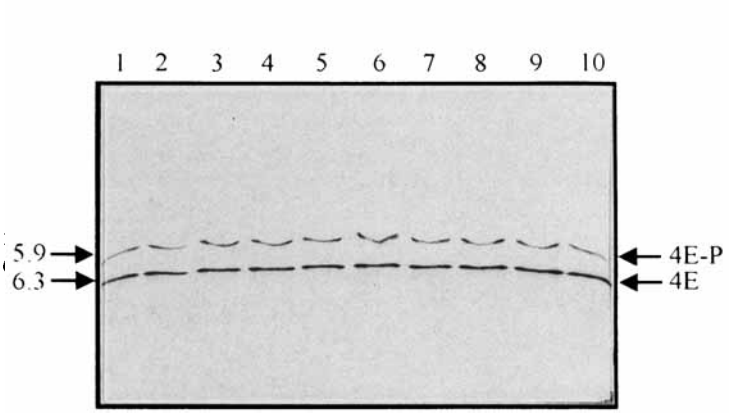
Differentiation Induced by Different Agents

The induction of differentiation in P19 cells with RA did not lead to a change in the eIF-4E phosphorylation state, although protein synthesis was increased transiently (Fig. 1). This result indicated that eIF-4E phosphorylation may not be a prerequisite for cellular differentiation, a conclusion that may be derived from the results of NGF addition to PC12 cells [Frederickson et al., 1992]. The lack of inducing eIF-4E phosphorylation in P19 cells during differentiation may be due to the absence of a component of the signal transduction pathway leading to eIF-4E phosphorylation. This question was addressed by using the P19-derivatives, expressing the human EGF-receptor (P19-EGF cells) with EGF [den Hertog et al., 1991a]. Depending on the

TABLE I. The Effect of Retinoic Acid and Serum on the Phosphorylation State of eIF-4E and on Translation Initiation in P19 Cells

	Duration of serum-deprivation (h)[a]									
	0		1.5		16		24		40	
Addition	T	4E-P	T	4E-P	T	4E-P	T	4E-P	T	4E-P
None	100	30	90	30	30	30	23	30	15	12
RA[b]	100	28	90	33	45	34	34	32	23	12
Serum[b]	100	ND	ND	ND	50	32	35	26	24	10

[a]Protein synthesis was measured after TCA precipitation (in %). The cells were labeled with ^{35}S-methionine/cysteine during 15 min before harvesting as described in the text. The phosphorylation state of eIF-4E was examined as described in Materials and Methods, and is given as percentage of phosphorylated eIF-4E over the total amount of eIF-4E.
[b]Lysates were prepared after 30 min of induction.



serumcontrol	+									
addition of serum		+								
" RA			+	+	+	+	+	+	+	+
incubation (minutes)		30	0	20	30	45	60	90	120	240
protein synthesis (%)	430	150	100	90	150	130	160	100	110	110
eIF-4E-P (%)	30	29	31	30	32	38	29	30	26	23

Fig. 1. Time-course of RA treatment of P19 cells. P19 cells were deprived of serum for 24 h. The cells were treated with serum for 30 min (*lane 2*) or with RA (10^{-6} M) for up to 4 h (*lanes 3–10*). The sample from serum-fed cells is in *lane 1*. Protein synthesis was measured by [^{35}S]methionine/cysteine incorporation, and lysates were run on a 1D iso-electric focusing gel. eIF-4E was detected by western blotting and the amount of phosphorylated eIF-4E was quantified by densitometry. The pH of the gel is indicated on the left.

culture conditions, P19-EGF cells respond to EGF addition by differentiation, in contrast to the parental P19 cells [den Hertog et al., 1991a]. Therefore, these cells can be used to determine whether the signal transduction pathway leading to eIF-4E phosphorylation is present in undifferentiated P19 cells.

Treatment of P19-EGF cells with EGF (Fig. 2, lanes 1–3) led to a 20% increase of protein synthesis in the first hour. The ratio of phosphorylated to unphosphorylated eIF-4E changed from 1:5.7 (Fig. 2, lane 1) to 1:1.6 (lane 3), a 3.6-fold increase.

P19-EGF cells were also treated with RA to show whether the expression of the EGF receptor influenced the effect of RA on eIF-4E phosphorylation. P19-EGF cells responded to RA similar to P19 cells, which shows that the EGF receptor did not change the response to RA. P19 cells were also treated with EGF. As expected from literature data [den Hertog et al., 1991a], P19 cells did not respond to EGF (data not shown).

Treatment of PC12 cells with the growth and differentiation agent NGF caused eIF-4E phosphorylation [Frederickson et al., 1992]. We repeated the experiment after a serum deprivation of 2 h, and measured protein synthesis and the eIF-4E phosphorylation state (Fig. 2, lanes 4–6).

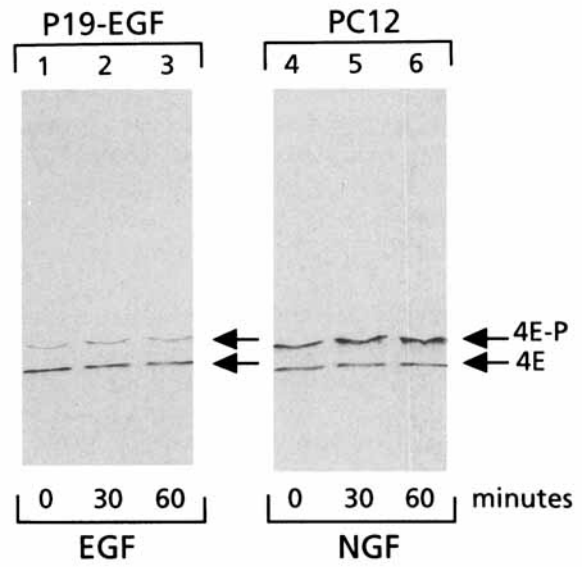


Fig. 2. Phosphorylation of eIF-4E in P19-EGF and PC12 cells. P19-EGF cells were deprived of serum and treated with EGF (50 ng/ml) for 0 (*lane 1*), 30 (*lane 2*), and 60 min (*lane 3*). PC12 cells were deprived of serum and treated with NGF (30 ng/ml) for 0 (*lane 4*), 30 (*lane 5*), and 60 min (*lane 6*). The cells were labeled with [^{35}S]methionine/cysteine for 30 min before harvesting, and the incorporation of [^{35}S]methionine/cysteine into protein was detected by trichloroacetic acid precipitation. Cells were harvested in sample buffer and run on a 1D iso-electric focusing gel. **Left:** Lane 1, 15% phosphorylated eIF-4E; lane 2, 21%; and lane 3, 38%. **Right:** Lane 4: 47% phosphorylated eIF-4E, lane 5: 60%, and lane 6: 70%.

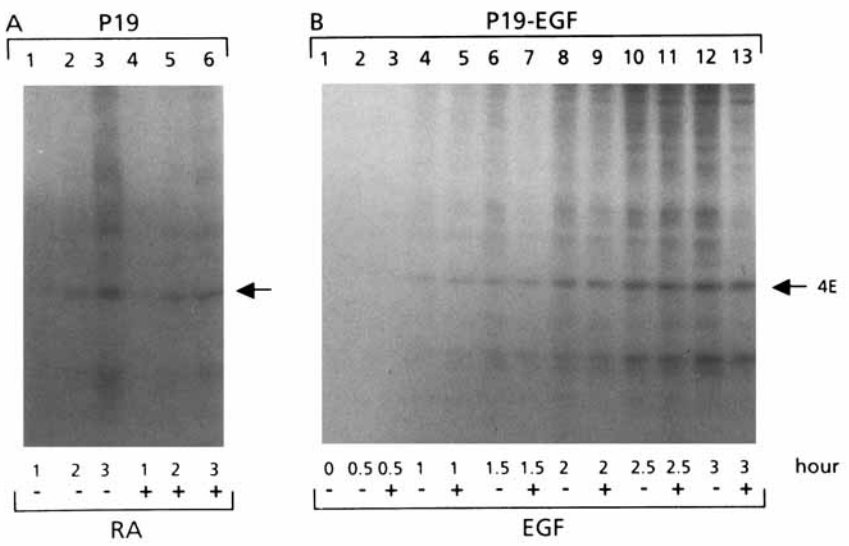


Fig. 3. [^{32}P]Orthophosphate labeling of P19 and P19-EGF cells. **A:** Cells were starved for serum for 16 h before the cells were labeled with [^{32}P]orthophosphate. The cells were labeled in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of RA. Cells were harvested after 1, 2, and 3 h. **B:** P19-EGF cells were deprived of serum for 4 h. The cells were labeled with [^{32}P]orthophosphate for 30 (*lanes 2 and 3*), 60 (*lanes 4 and 5*), 90 (*lanes 6 and 7*), 120 (*lanes 8 and 9*), 150 (*lanes 10 and 11*), and 180 min (*lanes 12 and 13*) in the absence or the presence of EGF. Cells were harvested and eIF-4E was purified with 7m GTP-Sepharose and analyzed by SDS-PAGE. Quantification of phosphate incorporation into eIF-4E was measured with the Phospho-imager (arbitrary units). A: Lane 1, 58,491; lane 2, 160,872; lane 3, 354,360; lane 4, 77,057; lane 5, 197,262; lane 6, 285,545. B: Lane 1, 0; lane 2, 4,400; lane 3, 7,510; lane 4, 55,321; lane 5, 57,919; lane 6, 64,314; lane 7, 58,960; lane 8, 155,625; lane 9, 136,615; lane 10, 212,380; lane 11, 230,998; lane 12, 268,427; lane 13, 228,773.

The rate of protein synthesis increased 50% in the first hour and the percentage of phosphorylated eIF-4E changed from 47% to 70% (Fig. 2, lanes 4 and 6), which means that the ratio of phosphorylated eIF-4E to the unphosphorylated form increased from 0.9 to 2.3, a 3-fold increase.

The results so far indicate that phosphorylation of eIF-4E occurred in PC12 cells stimulated with NGF, and P19-EGF cells stimulated with EGF. Phosphorylation of eIF-4E in P19-EGF cells upon EGF treatment showed that the signal transduction pathway involved in eIF-4E phosphorylation is present in undifferentiated P19 cells. However, the results suggest that this signal transduction pathway is not used during RA-induced differentiation. In spite of this differential effect on the eIF-4E phosphorylation state, protein synthesis increased in all three cases. Synthesis of eIF-4E, measured by [^{35}S]methionine/cysteine labeling, did not change after RA or EGF treatment (data not shown). Therefore, the increase in protein synthesis cannot be caused by the presence of an increasing amount of eIF-4E. The tentative conclusion is that translation initiation in P19 cells is not only regulated by the phosphorylation state of eIF-4E. However, it is possible that the turnover of phosphate on eIF-4E is increased after RA treatment of P19 cells, without changing the phosphorylation state. Two examples already exist in which turnover is increased severalfold, while the phosphorylation state is hardly changed [Rinker-Schaeffer et al., 1992; Rychlik et al., 1990].

Incorporation of [^{32}P]orthophosphate Into eIF-4E in P19 and P19-EGF Cells in the Absence and Presence of an Inducer

To determine whether RA treatment changed the turnover of phosphate on eIF-4E, P19 cells were labeled with [^{32}P]orthophosphate. Also, P19-EGF cells were labeled with [^{32}P]orthophosphate in the absence and the presence of EGF to determine whether the phosphorylation or dephosphorylation rate of eIF-4E was responsible for the increase in the phosphorylation state of eIF-4E as shown in Figure 2.

The kinetics of phosphate labeling of eIF-4E in P19 cells were similar in the absence and the presence of RA (Fig. 3A). Apparently, the rate of eIF-4E phosphorylation did not change after RA addition.

The amount of phosphorylated eIF-4E in EGF-treated P19-EGF cells was similar to that of

control cells (Fig. 3B). As the eIF-4E phosphorylation state in P19-EGF cells increased after EGF treatment (Fig. 2), the results indicate that the rate of dephosphorylation of eIF-4E is influenced by EGF.

Dephosphorylation of eIF-4E After Induction of Differentiation

P19 cells and P19-EGF cells were deprived of serum and supplemented during the last 3 h of the serum deprivation with [^{32}P]-labeled orthophosphate. After the labeled medium was removed, cells were treated with RA or EGF for the times indicated in Figure 4.

The amount of [^{32}P]-labeled phosphate on eIF-4E in P19 cells decreased in the control cells, as well as in the RA treated cells in a similar way (Fig. 4). The half-life of the phosphate on eIF-4E in the absence and in the presence of RA was about 41 min. As expected from the previous results, the rate of eIF-4E dephosphorylation was not influenced by the addition of RA.

The half-life of a phosphate on eIF-4E in untreated P19-EGF cells (Fig. 4, lanes 2–4) was similar to the one found in P19 cells (41 min). In contrast, the amount of [^{32}P]-labeled eIF-4E did not decrease but even increased 1.6-fold, when P19-EGF cells were chased in the presence of EGF (Fig. 4, lanes 12–14). This result together with the changed phosphorylation state and the unchanged eIF-4E phosphorylation rate indicates that EGF modulates eIF-4E dephosphorylation.

During a chase of P19-EGF cells after [^{32}P]orthophosphate labeling in the absence and the presence of RA, the same results were obtained as shown for P19 cells (data not shown).

DISCUSSION

P19 cells were treated with the differentiation agent RA to measure whether differentiation coincided with a change in the rate of protein synthesis and eIF-4E phosphorylation. RA treatment led to an increase in protein synthesis within 30–60 min (Table I). However, eIF-4E phosphorylation state (Fig. 1), the turnover of phosphate on eIF-4E (Figs. 3 and 4), and the synthesis of eIF-4E (data not shown) remained the same. These results show that the very early onset of differentiation of P19 cells does not require changes in eIF-4E phosphorylation.

RA can influence cell growth [Sporn and Roberts, 1983], pattern formation in limb development and regeneration [Maden, 1982; Takaha-

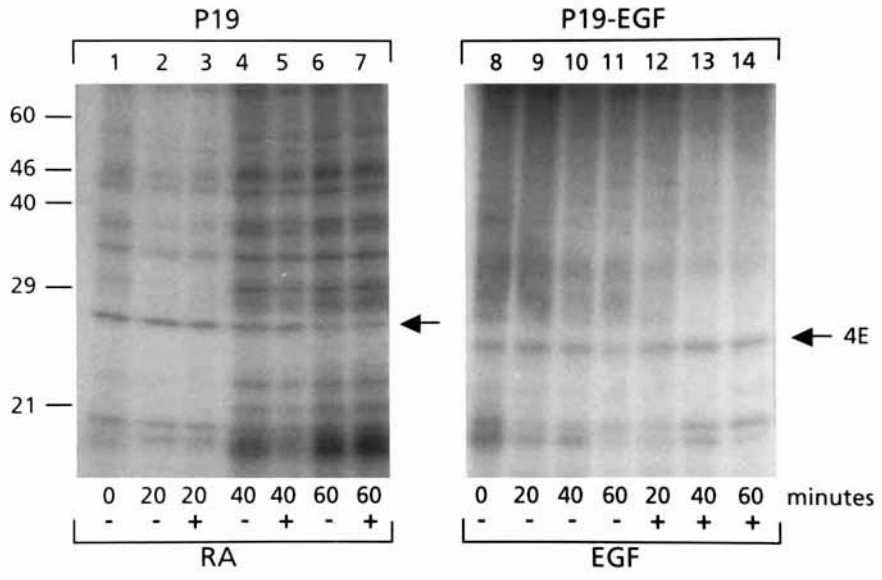


Fig. 4. Turnover of phosphate on eIF-4E in P19 and P19-EGF cells. P19 cells and P19-EGF cells were starved for serum. During the last 3 h, [^{32}P]-labeled orthophosphate was added. Label was removed and the cells were chased in the absence or presence of RA (P19) (**left**) or EGF (P19-EGF) (**right**). P19 cells were harvested after 0 (*lane 1*), 20 (*lanes 2 and 3*), 40 (*lanes 4 and 5*), and 60 min (*lanes 6 and 7*). P19-EGF cells were harvested after 0 (*lane 8*), 20 (*lanes 9 and 12*), 40 (*lanes 10 and 13*), and 60 min (*lanes 11 and 14*). eIF-4E was purified and analyzed by SDS-PAGE. Incorporation of phosphate into eIF-4E was measured with a Phospho-imager (arbitrary units). Lane 1, 201,484; lane 2, 148,409; lane 3, 148,729; lane 4, 120,292; lane 5, 103,310; lane 6, 71,873; lane 7, 96,418; lane 8, 216,598; lane 9, 232,557; lane 10, 183,833; lane 11, 96,566; lane 12, 235,042; lane 13, 314,975; lane 14, 309,956.

shi et al., 1975], and fetal development [Soprano et al., 1986; Tickle et al., 1985]. Furthermore, RA activates diacylglycerol, and causes a translocation of PKC from the cytosol to the membrane within minutes. This suggests a rapid signal transduction from the nuclear RA-receptor to the cytoplasm [Kurie et al., 1993], which is supported by the fast increase in protein synthesis after RA addition (Table I).

Increased protein synthesis in RA-treated cells cannot be explained by increased elF-4E phosphorylation, and regulation of other initiation factors must be involved. One possible explanation for increased protein synthesis is by regulation of the recently discovered 4E-BP1 or PHAS-I protein [Pause et al., 1994]. Phosphorylation of 4E-BP1 influenced the activity of elF-4E. The effect of elF-4E phosphorylation on the regulation by 4E-BP1 was not determined, and 4E-BP1 regulation would not require elF-4E phosphorylation per se. It was suggested that 4E-BP1 phosphorylation dissociates the 4E-BP1-elF-4E complex and liberates elF-4E, enabling cap binding and initiation of protein synthesis [Pause et al., 1994].

To measure whether the unchanged elF-4E phosphorylation state was due to the absence of the signal transduction pathway leading to elF-4E phosphorylation in undifferentiated P19 cells, P19-EGF cells were treated with EGF. Upon EGF treatment an increase in the phosphorylation state of elF-4E occurred (Fig. 2). Therefore, the signal transduction pathway is present in undifferentiated P19 cells. However, this pathway was not used when P19 cells were differentiated with RA. Apparently, elF-4E phosphorylation is not an absolute prerequisite to induce differentiation after RA-addition.

Undifferentiated P19 cells do not express detectable levels of epidermal growth factor receptors (EGF-R). However, after 2 days of RA-induced differentiation increased levels of EGF-R mRNA and protein has been found [Joh et al., 1992]. Reduced expression of EGF-R in differentiating P19 cells inhibited the ability to differentiate [Wu and Adamson, 1993]. Undifferentiated P19 cells overexpressing the EGF receptor could be stimulated to differentiate upon EGF treatment when grown in aggregates. However, under our cell culture conditions—growing the cells in monolayers—differentiation does not occur upon EGF addition [den Hertog et al., 1991a]. Therefore, the increase in protein synthesis and elF-4E phosphorylation as shown in Figure 2 was due to growth stimulation.

Besides having an effect on differentiation, NGF can stimulate proliferation of PC12 cells [Boonstra et al., 1983]. Therefore, the effect of NGF addition on elF-4E phosphorylation in PC12 cells could be explained by the growth stimulatory effect of NGF. Treatment of P19-EGF cells with the growth inducer EGF also led to elF-4E phosphorylation and increased protein synthesis (Fig. 2). This indicates that elF-4E phosphorylation coincides with growth stimulation.

Treatment of P19-EGF cells with EGF increased the phosphorylation state of elF-4E (Fig. 2). This increase was solely due to a decreased dephosphorylation rate and not due to modulation of the elF-4E phosphorylation rate (Figs. 3 and 4). This result indicates that an elF-4E phosphatase is influenced after EGF treatment. Regulation of the elF-4E phosphorylation state by phosphatase activity has been described before. Treatment of human mammary epithelial cells (184N1A4) with EGF led to an elevation of the elF-4E phosphorylation state of 2–3-fold [Donaldson et al., 1991]. Okadaic acid, a potent inhibitor of phosphatase 1 and 2A, also led to an increase in elF-4E phosphorylation. Treatment of the cells with EGF and okadaic acid simultaneously did not result in a further elevation of elF-4E phosphorylation [Donaldson et al., 1991]. This may suggest that both EGF and okadaic acid act on the same phosphatase.

The turnover of phosphates on elF-4E was similar in P19 and P19-EGF cells. The half-life of the phosphate on elF-4E was about 41 min. Zhang et al. [1994] described that the half-life of a phosphate on elF-4E in 293 cells was 32–36 min, a value similar to the one found here with P19 cells. Although only based on two observations, this finding suggests that the turnover rate of phosphorylated elF-4E is similar in different cell types.

Treatment of PC12 cells with NGF led to a 4-fold increase in the amount of phosphorylated elF-4E [Frederickson et al., 1992]. Treatment of 184N1A4 cells with EGF led to a 2–3-fold increase in elF-4E phosphorylation [Donaldson et al., 1991]. The ratio of phosphorylated elF-4E to the unphosphorylated form was 0.17 in control P19-EGF cells, and 0.61 in EGF-treated cells, a 3.6-fold increase (Fig. 2). The total amount of EGF receptors on 184N1A4 cells (300,000 receptors/cell) [Donaldson et al., 1991] is 10-fold higher than the amount of EGF receptors on P19-EGF cells [den Hertog et al., 1991b], and PC12 cells expose 58,000 NGF receptors on

their surface [Herrup and Thoenen, 1979]. The difference in receptor type and in the amount of receptors does not influence the extent of the stimulation of the eIF-4E phosphorylation.

The retinoic acid receptor is a nuclear receptor and a member of the steroid/thyroid hormone receptor family [Giguere et al., 1987; Petkovich et al., 1987]. EGF and NGF both bind to a membrane bound tyrosine kinase receptor and have the ability to increase protein synthesis as well as to increase eIF-4E phosphorylation. RA induces the cell via a nuclear receptor and increased protein synthesis only. Apparently the nuclear signal is transmitted to the cytoplasmic translational apparatus. The result may imply that an increase in eIF-4E phosphorylation occurs after activation of receptor tyrosine kinases or after activation of one of the intermediates of the tyrosine kinase signal transduction pathway.

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