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Phosphorylation of Ribosomal Protein S6 and its Regulation During Differentiation of Human Leukemic Cells

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We attempted to study the role of protein tyrosine kinase (PTK) and protein kinase C (PKC) in the cascade of phosphorylation of ribosomal protein S6 during differentiation of leukemic cells (HL-60, THP-1, and RWLeu-4). Neither activation nor inhibition of colony stimulating factor-1 (CSF-1) receptor's PTK activity with CSF-1 or genistein respectively affected the phosphorylation of S6. However, vanadate which is a protein tyrosine phosphatase (PTP) inhibitor showed enhancement of S6 phosphorylation. Dimethylsulfoxide which does not affect either PTK or PKC demonstrated no change in S6 phosphorylation.

PKC activation by acute 12-0-tetradecanoyl phorbol-13-acetate (TPA) treatment induced monocytic differentiation and S6 phosphorylation. Surprisingly, the more prominent phosphorylation of S6 protein was observed in PKC-depleted cells by prolonged TPA treatment.

Our results suggest that PTK/PTP play a lesser role in S6 phophorylation of HL-60 cells than PKC does. In addition, two different mechanisms seem to be involved in TPA-induced S6 phosphorylation during HL-60 differentiation: PKC activation by acute TPA treatment and PKC depletion which may lead to the synthesis of some endogenous protein responsible for the differentiation by chronic TPA treatment.

Key Words: *Differentiation, S6, TPA, Protein kinase C, Protein tyrosine kinase*

INTRODUCTION

At a certain point of cell replication, normal cells stop growing and differentiate into cells that have specific functions. However, tumor cells differ from normal cells in that they are no longer responsive to normal growth controlling mechanisms. A dedifferentiation or block to normal differentiation often accompanies malignant transformation of cells (Pierce and Speers, 1988).

The continuous growth of tumor cells can be blocked and induced to differentiate by certain differentiation-inducing agents such as retinoic acid, dimethylsulfoxide (DMSO), vitamin D_3, and 12-O-tetradecanoyl phorbol-13-acetate (TPA)(Yen et al., 1987; Birnie, 1988). Among these differentiation agents, retinoic acid and vitamin D_3 have been used for the therapy or chemoprevention of some human tumors (Meeting report on 'Differentiation therapy', 1990). TPA is the most widely used experimental tool as an activator of protein kinase C (PKC) by acute treatment . It is also well known that PKC is depleted by prolonged treatment of TPA (Blumberg, 1988). There have been reports showing that TPA differentiates melanoma cells to melanocytes, and promyelocytic HL-60 and monocytic THP-1 leukemic cells to monocytes (Huberman et al., 1979; Frank and Sartorelli, 1986; Auw-

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erx, 1991). Other than PKC, in many eukaryotic cells protein tyrosine kinase (PTK) has been suggested to play an important role in cell transformation and differentiation (Hunter and Cooper, 1985; Yarden and Ullrich, 1988). Thus, it is very interesting to ask how these two enzyme systems affect each other in normal monocytes and in leukemic cells (HL-60, THP-1, and RWLeu-4) which are inducible to mature.

Not only PKC activators but also various PTK activators and protein tyrosine phosphatase (PTP) inhibitors are known to stimulate S6 kinase which phosphorylates 40S ribosomal protein S6 on serine residues. Phosphorylation of S6 apparently increases the affinity of the 40S ribosome for messenger RNA. This results in elevated levels of protein synthesis followed by cell proliferation or differentiation (Thomas, 1986). Therefore, in this study, phosphorylation of S6 has been chosen as a hallmark to investigate the involvement of PKC and PTK in the differentiation mechanism of HL-60 cells.

MATERIALS AND METHODS

Materials

12-O-tetradecanoyl phorbol-13-acetate (TPA), dimethylsulfoxide (DMSO), and sodium orthovanadate (vanadate) were obtained from Sigma Chemical Co, USA. Genistein was purchased from ICN, USA. TPA and genistein were dissolved in DMSO and diluted in the culture medium (Final concentration of DMSO was not over 0.01%). ^{32}P-orthophosphate and ^{35}S-methionine were bought from Amersham, UK, and recombinant human colony stimulating factor-1 (CSF-1) was donated by Dr. P. Ralph.

Cell culture

HL-60 (human acute promyelocytic leukemia), RWLeu-4 (human chronic myelogenous leukemia), and THP-1 (human monocytic leukemia) cells were grown in RPMI-1640 (GIBCO, USA) supplemented with non-essential amino acids and 1 mM sodium L-pyruvate, 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS, GIBCO, USA) in 5% CO_2/95% air at 37 °C.

Isolation of human peripheral blood monocytes

Platelet-free blood monocytes were isolated by sedimentation on a cushion of Histopaque-1077 (Sigma, USA), followed by sequential washes in Ca^{++}-free, Mg^{++}-free phosphate buffered saline (PBS) containing 0.3 mM EDTA and in serum containing 5 mM EDTA, and plastic adherence in tissue culture dishes as described by Pawlowski et al. (1983).

Pulse-chase labeling with ^{35}S-methionine

HL-60 cells were grown to be 7×10^5 cells/well in a 6-well plate with RPMI-1640 containing 10% FCS and were treated with 10 ng/ml (17 nM) of TPA for 3 days. The control cells were treated with 0.01% DMSO in the medium which is the composition of solvent for TPA. The cells were then methionine-depleted for 30 min in methionine-free medium before 20 min pulse of ^{35}S-methionine labeling (0.1 mCi/ml). Incorporated ^{35}S-methionine was then chased using media containing 40 mM methionine for 0, 0.5, 3, 12, and 24 hours each.

Both control and TPA-treated cells were washed with PBS and lysed with S6 lysis buffer. The supernatant after centifugation at 13,000×g for 10 min was used for the trichloracetic acid-precipitated protein determination and for further analysis of ribosomal protein S6.

Serine phosphorylation of ribosmal protein S6

HL-60 cells were grown to be 3×10^5/well in a 12-well plate with RPMI-1640 containing 10% FCS. The cells were then treated with 10 ng/ml(17 nM)of TPA for 3 days to deplete PKC and 10 ng/ml genistein for 24 hours to inhibit PTK. For the differentiation of HL-60 cells into granulocytes without affecting PKC or PTK activity, 1.2% DMSO was added for 3 days. All the control, TPA-, genistein-, and DMSO-treated cells were washed twice with phosphate-free or methionine-free DME medium and incubated with either ^{32}Pi (0.1 mCi/ml) or ^{35}S-methionine(0.1 mCi/ml) for 3 hours at 37°C. To activate PKC, 10 ng/ml TPA was added for 10 min, to activate PTK, 2000 U/ml CSF-1 was added for 30 min, and to inhibit PTP, 1 mM vanadate was added for 30 min during each incubation period. These cells were then washed twice with ice-cold PBS, and solubilized with S6 lysis buffer : 1ml of ice-cold 50 mM Pipes (pH 7.5), 5 mM $MgCl_2$, 5 mM KCl, 50 mM NaF, 40μM EDTA, 1% (wt/vol) deoxycholate, 1% (wt/vol) Triton X-100 and 1 mM PMSF. Cell extracts were clarified by microcentrifugation (13,000×g for 10 min) and layered over 2.0 ml of 1.6 M sucrose, 50 mM Pipes (pH 7.5), 5 mM $MgCl_2$, 0.5 M KCl, 40μM EDTA, and the ribosomes were pelleted by microcentrifugation (160,000×g at 4°C for 2 hours). The ribosomal pellet was dissolved in

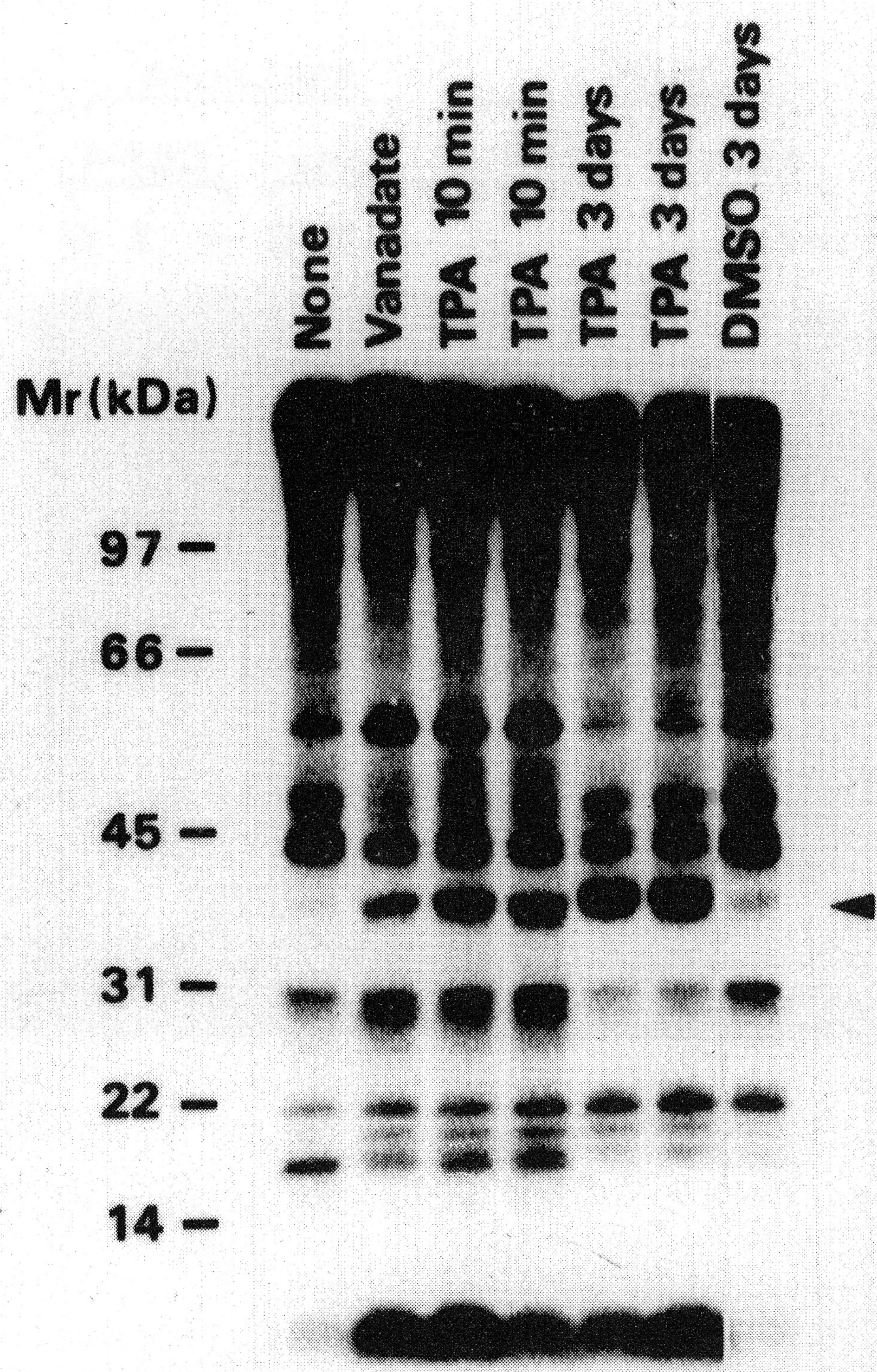

Fig. 1. Effect of vanadate, TPA, and DMSO on S6 phosphorylation in HL-60 cells. TPA 10 ng/ml, DMSO 1.2%. The arrow indicates 33-kDa ribosomal protein S6 in ^{32}Pi-labeled cells. Five independent experiments were performed.

200μl Laemmli sample buffer containing 2 mM dithiothreitol and 2% β-mercaptoethanol, and boiled. The S6 protein was identified by electrophoresis on SDS gels containing 10% acrylamide, and visualized by autoradiography. Phosphoserine was the only phosphoamino acid detected in the 33-kDa S6 protein (data not shown). The 33-kDa ribosomal protein S6 band was cut out from the gel for the quantitation of its radioactivity with a β-counter.

RESULTS

Effect of differentiation on S6 phosphorylation in HL-60, THP-1, and RWLeu-4 cells

The PKC activator, TPA, has been known for years to induce HL-60 cells to differentiate into monocytes (Meeting report on 'Differentiation therapy', 1990). In our studies, TPA-treated HL-60 cells also stopped proliferating and became adherent to plastic surfaces which are characteristics of monocytes. First, the effect of two differentiation inducing agents, TPA and DMSO, on the phosphorylation of ribosomal protein S6 was tested in HL-60 cells. There was a 9.3 fold increase with 10 min treatment of TPA and a 16 fold increase with 3 days treatment of TPA in the phosphorylation of this protein. However, DMSO which induces granulocytic differentiation of HL-60 cells did not change the phosphorylation of S6. The vanadate, a PTP inhibitor (Swarup et al., 1982; Yonemoto et al., 1987), showed 5.4 fold increase of S6 phosphorylation (Fig. 1 & Table 1).

Table 1. Effect of vanadate, TPA, and DMSO on S6 phosphorylaton in HL-60 cells.

Treatment	Counts(cpm)	% Change
None	282	100
Vanadate	1528	542
TPA 10 min	2670	947
TPA 10 min	2576	913
TPA 3 days	4241	1504
TPA 3 days	4789	1698
DMSO 3 days	305	108

The 33-kDa ribosomal S6 band in Fig. 1 was cut out from the gel for the quantitation of its radioactivity with a β-counter.

To compare the S6 responses in normal monocytes and in differentiated monocytes from HL-60 cells, TPA was added to both cells for 3 days. It clearly showed the marked phosphorylation of S6 by TPA in both cells. We also were able to confirm the reduction of PKC activity in the TPA-treated cells for 3 days to be 5% of non-treated cells (data not shown). Monocytes are known to be proliferating and differentiating from the precursor cells when stimulated with CSF-1 (Whetton AD, 1990). CSF-1 binds to its CSF-1 receptor on monocytes which results in the activation of the receptor's PTK and affects the signal transduction leading to cell proliferation and differentiation (Huhn et al., 1989). To study the role of PTK in the differentiation of HL-60 cells, either a PTK activator, CSF-1, or a PTK inhibitor, genistein (Akiyama et al., 1987), was added to both monocytes and HL-60 cells. Neither of the agents affected the phosphorylation of S6 (Fig. 2; lane 3, 4, 6, 9, 10 & 12).

Second, we thought it was very important to investigate the interaction of PTK and PKC in the pathways leading to S6 phosphorylation. In PKC-

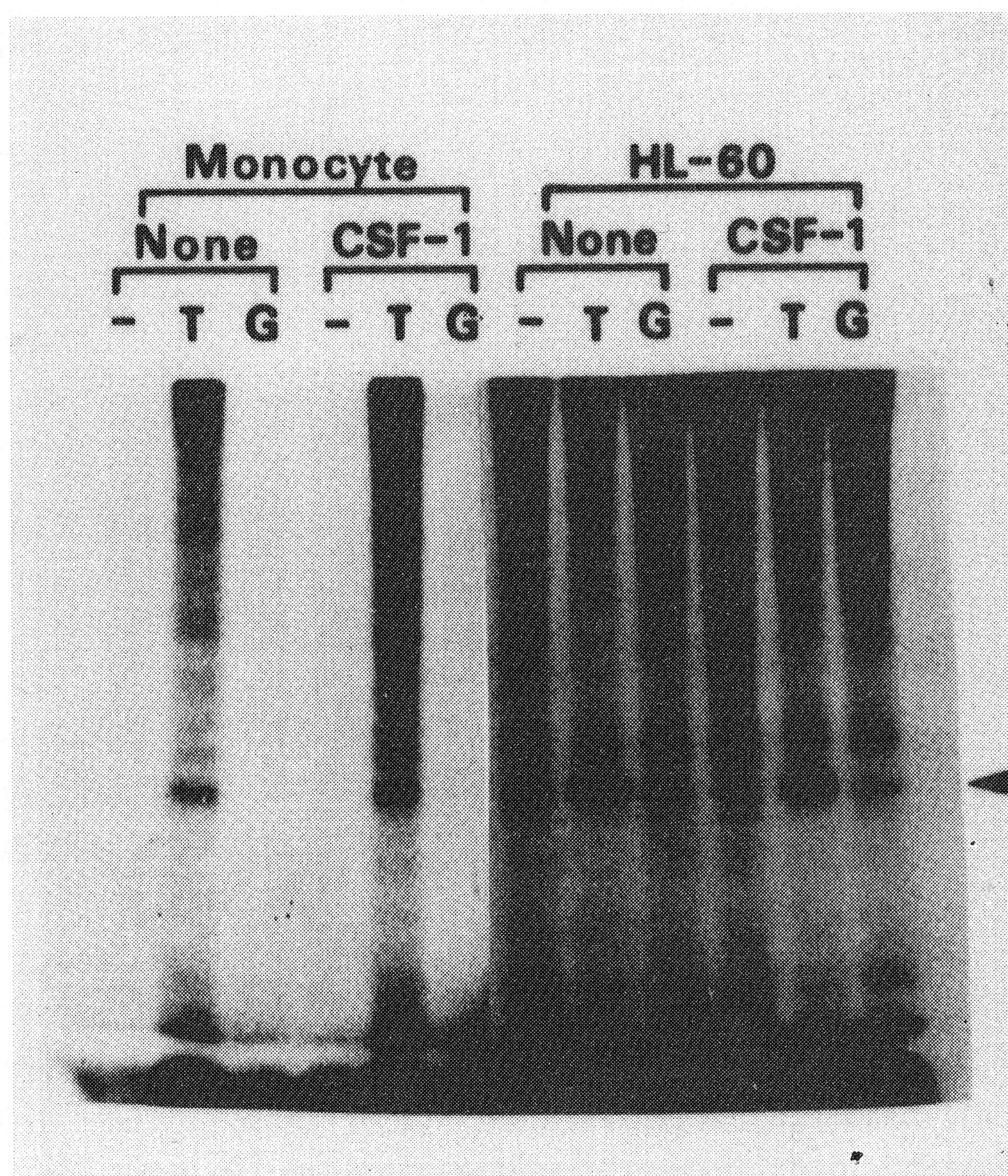

Fig. 2. Effect of TPA, genistein, and CSF-1 on S6 phosphorylation in monocytes and HL-60 cells. -; control (0. 01% DMSO in RPMI-1640), T; TPA 10 ng/ml for 3 days, G; genistein 10 ng/ml for 24 hours, CSF-1; 2000 U/ml for 30 min. The arrow indicates 33-kDa ribosomal protein S6 in ^{32}Pi-labeled cells. Three independent experiments were performed.

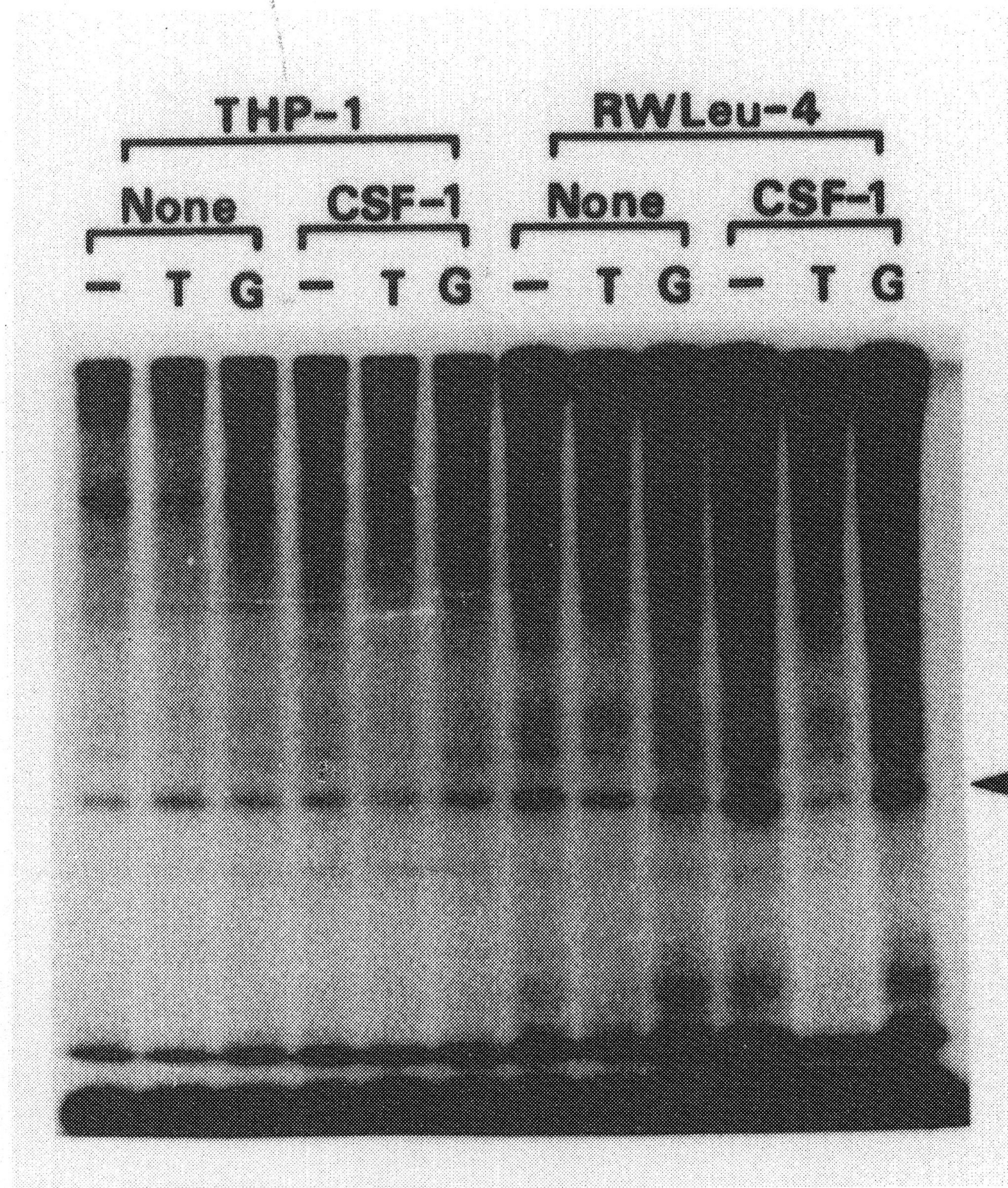

Fig. 3. Effect of TPA, genistein, and CSF-1 on S6 phosphorylation in THP-1 and RWLeu-4 cells. -; control (0. 01% DMSO in RPMI-1640), T; TPA 10 ng/ml for 3 days, G; genistein 10 ng/ml for 24 hours, CSF-1; 2000 U/ml for 30 min. The arrow indicates 33-kDa ribosomal protein S6 in ^{32}Pi-labeled cells. Four independent experiments were performed.

depleted cells, activating PTK of CSF-1 receptor with CSF-1 did not induce any further change in S6 phosphorylation either in monocytes or HL-60 cells (Fig. 2; lane 2 & 5, lane 8 & 11).

To compare the difference of the differentiation response between malignant (THP-1 & RWLeu-4) and less malignant (HL-60) cells, S6 phosphorylation in TPA-treated cells was studied. In contrast to the TPA effect in HL-60 cells, we could not see any change of S6 phosphorylation in either THP-1 or RWLeu-4 cells with any of the agents tested; TPA, genistein, and CSF-1 (Fig. 3).

Turnover of S6 protein in HL-60 cells

In view of the fact that PKC is activated by TPA, the 9.3 fold increase of S6 phosphorylation was expectable in 10 min of TPA-treated HL-60 cells. However, the 16 fold increase of S6 phosphorylation in PKC-depleted cells by 3 days treatment of TPA was a surprising result since we were expecting rather a decrease of S6 phosphorylation. In addition to the mechanism for the S6 response in proliferating cells, there may well be another mechanism for this response in differentiating cells. As a mechanism, there may be some protein needed for the differentiation of HL-60 cells. Then there should be either quantitative increase of ribosomal protein S6 or qualitative increase in phosphorylation of S6 for the synthesis of this unknown protein responsible for the differentiation. To pursue this issue, we did a pulse-chase labeling experiment using ^{35}S-methionine which is a classical standard procedure for the study of protein turnover. The turnover rates of 33-kDa S6 protein in both control and TPA-treated cells were about the same ; 20%~30% increase at 0.5 hours, maximum at 3 hours, and reduction to 50% of maximum at 12 hours (Fig. 4 & Table 2).

DISCUSSION

To examine the involvement of PTK in the signal transduction pathway to S6 phosphorylation in monocytes, HL-60, THP-1, and RWLeu-4 cells we tried to stimulate or inhibit the PTK of CSF-1 receptor with CSF-1 or genistein respectively. Neither of these agents affected the phosphorylation of S6. Both non-differentiated and differentiation-induced

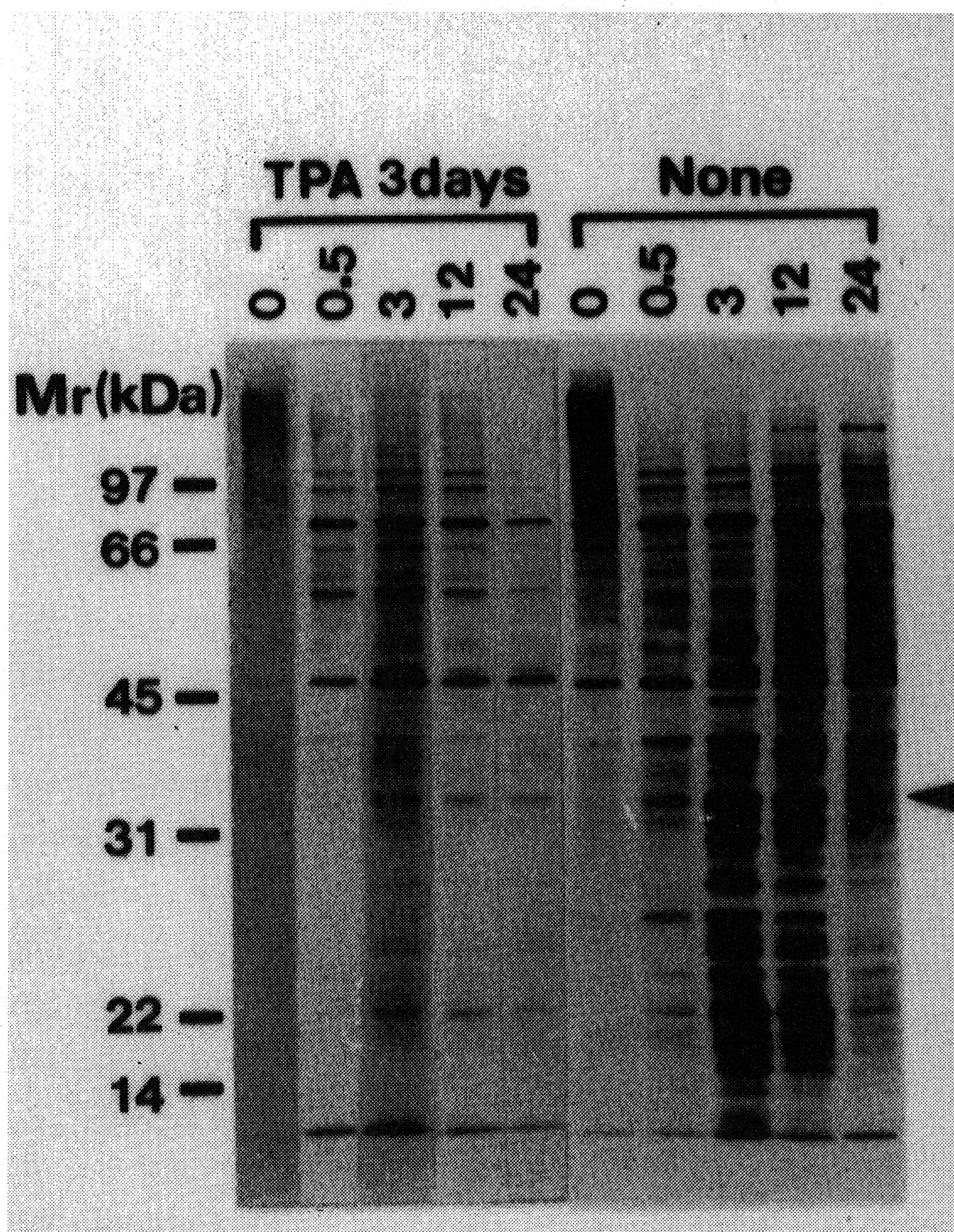

Fig. 4. Effect of TPA on the synthesis and degradation of ribosomal protein S6 in HL-60 cells. The numbers (0, 0.5, 3, 12, 24) are chasing hours with 40 mM methionine. TPA 10 ng/ml. The arrow indicates 33-kDa ribosomal protein S6 in ^{35}S-methionine-labeled cells. Three independent experiments were performed.

Table 2. Effect of TPA on the synthesis and degradation of ribosomal protein S6 in HL-60 cells.

Chasing period	% of Basal level	
(hour)	None	TPA
0	100	100
0.5	124	131
3	1347	1426
12	686	733
24	454	492

The 33-kDa ribosomal S6 band in Fig. 4 was quantitated by densitometry. Basal level; amount of S6 phosphorylation at O hour.

HL-60 cells with TPA did not respond differently either to CSF-1 or genistein. However, these results do not necessarily mean that PTK has no role in S6 phosphorylation in these cells. Although genistein has been reported to inhibit PTK of epidermal growth factor, pp60$^{v\text{-}src}$, and pp 110$^{gag\text{-}fes}$ selectively (Akiyama et al., 1987), it may not inhibit the PTK of CSF-1 receptor. As evidence for the involvement of PTK in this S6 response, vanadate, which prolongs PTK activity by inhibiting PTP, induced a 5 fold increase of S6 phosphorylation. Vanadate could inhibit PTP working at any point in the S6 pathway. In addition, DMSO, the differentiating agent which affects neither PTK nor PKC, did not show any change in S6 phosphorylation. There are a couple of reports demonstrating that the increased total PTK activity is counteracted by the greater elevation of PTP activity during HL-60 differentiation (Frank and Sartorelli, 1986; 1988). Hence, the end result shows overall increased PTP activity and these findings are supportive of our results that S6 phosphorylation increases only in vanadate-treated cells. Furthermore, our results suggest that PTK/PTP play a lesser role in S6 phosphorylation of HL-60 cells than PKC does.

To pursue the role of PKC in S6 phosphorylation, PKC was either activated or depleted by incubating HL-60 cells with TPA for 10 min or for 3 days respectively. As we expected from other reports (Trevillyan et al., 1984; Blenis et al., 1984), our results also showed that PKC activation by TPA 10 min treatment induced the distinct phosphorylation of S6. However, it is a contrasting phenomenon that PKC depletion by TPA 3 days treatment resulted in more extensive phosphorylation of S6 as we can see from its darker density of 33-kDa band and its slower migration on polyacrylamide gel electrophoresis. By extensive study and recent progress, the signaling cascade of S6 phosphorylation has been well established. Various growth factor receptors which have PTK activities upon their ligands binding, oncogene products, and PKC activators are known to activate S6 kinases which phosphorylate S6 on serine residues (Thomas, 1986). These S6 kinases themselves are not tyrosine phosphorylated, but appear to be regulated by other classes of kinases; the extracellular signal responsive kinases (ERKs). ERKs themselves are active only when coincidentally phosphorylated on both tyrosine and threonine residues (Cobb et al., 1991). ERKs either phosphorylate themselves in response to activator protein (Ahn et al., 1991), or are phosphorylated by another kinase (Gomez and Cohen 1991; L'Allermain et al., 1992). It is presumed that upstream of the ERKs (and similarly, upstream of S6 kinases) there must exist tyrosine phosphorylated substrate (s) of the growth factor receptors. Since PKC, a serine/threonine kinase, does not phosphorylate

either S6 protein or S6 kinases directly, it is presumed to work at some point upstream of S6 kinases in S6 cascades.

At present, we do not know the exact site of action of PKC in the S6 pathway. In addition, PKC activation is not sufficient for the induction of HL-60 differentiation since both TPA and diacylglycerol activate PKC, but only TPA is an effective differentiation inducer (Meeting report on 'Differentiation therapy', 1990). As one possible mechanism for the extensive phosphorylation of S6 by PKC depletion, there may be activation of some PTK rather than CSF-1 receptor by PKC depletion, thereby activating ERKs or the upstream of ERKs. Another very important and interesting explanation is that there may be some endogenous synthesis of differentiation-inducing protein(s) during differentiation of HL-60 cellls. In our pulse-chase experiment of S6 protein, we could see rather higher overall S6 phosphorylation in control cells than in TPA-treated cells at each time point of the chasing period. If we assume both control and TPA-treated cells have qualitatively the same amount of S6 phosphorylation, then it is difficult to interpret the marked increase of S6 phosphorylation in TPA-treated cells as shown in Fig. 1. To overcome the quantitatively enhanced synthesis of S6 protein seen in control cells (Fig. 4) there should be qualitatively much higher phosphorylation of S6 protein in TPA-treated cells. As evidence for this explanation, the mobility of 33-kDa S6 protein is much more retarded in the 3 days TPA-treated cells than in the control or the 10 min TPA-treated cells (Fig. 1), which means higher phosphorylation of S6 protein in the 3 days TPA-treated cells. This conclusion is based on the observation from others that the more highly phosphorylated S6 is, the more slowly it migrates in electrophoresis (Gressner and Wool, 1974; Thomas et al., 1979). This qualitative increase of S6 phosphorylation seems to occur before the production of some differentiation-inducing protein(s). So far, only two cytosolic proteins which are 17-kDa serine- and 75-kDa tyrosine-phosphorylated, have been found to be related to the differentiation of HL-60 cells (Braverman et al., 1986; Bushkin et al., 1991). We still do not know the roles of these proteins in S6 phosporylation. Further characterization of these proteins and discovery of yet unidentified protein (s) are required to elucidate the mechanisms of cell differentiation.

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