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Phosphorylation and *O*-Linked Glycosylation of Elf-1 Leads to Its Translocation to the Nucleus and Binding to the Promoter of the TCR ζ-Chain[1]

Yuang-Taung Juang,*† Elena E. Solomou,*† Barbara Rellahan,‡ and George C. Tsokos[2]*†

Elf-1, a member of the E 26-specific transcription factor family with a predicted molecular mass of 68 kDa, is involved in the transcriptional regulation of several hematopoietic cell genes. We demonstrate that Elf-1 exists primarily as a 98-kDa form in the nucleus and as an 80-kDa form in the cytoplasm. Phosphorylation and *O*-linked glycosylation contribute to the increased posttranslational molecular mass of Elf-1. The 98-kDa Elf-1 is released from the cytoplasm tethering retinoblastoma protein and moves to the nucleus, where it binds to the promoter of the TCR ζ-chain gene. Finally, the cytoplasmic 98-kDa form enters the proteasome pathway and undergoes degradation. In conclusion, different forms of Elf-1 are the products of posttranslational modifications that determine its subcellular localization, activity, and metabolic degradation. ***The Journal of Immunology,*** **2002, 168: 2865–2871.**

Elf-1 is a transcription factor that belongs to the E 26-specific (Ets) family of proteins. Members of the Ets family are expressed in hematopoietic cells and at lower levels in fibrocytic cells. In hematopoietic cells, Elf-1 mediates the induction of various groups of genes, including genes for the *blk*, *lck*, and *lyn* kinases (1); surface membrane proteins (TCR ζ-chain (2, 3), IL-2R α-chain (4), and CD4 (5)); and cytokines (GM-CSF (6) and IL-2 (7)). The contribution of Elf-1 to the transcriptional regulation of each gene may be variable. In the case of the TCR ζ-chain gene, point mutations, introduced into the two Elf-1 binding sites (−147/−119 and −66/−33) of the TCR ζ-chain promoter, abolish the basal transcriptional activity of the ζ-chain promoter in Jurkat cells (2, 3).

The Elf-1 cDNA (GenBank accession no. P32519) contains an open reading frame of 1,857 nt and therefore encodes a protein of 619 aa with an expected molecular mass of 68 kDa. However, most of the published work indicates that Elf-1 exists as a protein with an apparent molecular mass of 98 kDa, although 80-kDa and 68-kDa forms have also been mentioned (1, 8). Interestingly, this discrepancy in the apparent molecular mass is not limited to Elf-1 and characterizes other Ets family members. Nerf, an Ets family-related protein, has a calculated molecular mass of 58 kDa, but it migrates as a 69-kDa protein (1). Proteins that contain a high ratio of either positively or negatively charged amino acids are known to display discrepancies between the estimated molecular mass and that observed by SDS gel electrophoresis. However, Elf-1 does not contain a high ratio of charged amino acids, suggesting that charge alone cannot be responsible for the dichotomy between the predicted and observed molecular mass. Proteins undergo one or even multiple types of modifications after translation, which are responsible for the molecular mass added to that calculated from the gene structure. Although phosphorylation is one of the most common posttranslational modifications of proteins, acetylation, sulfation, and glycosylation are also involved (9). The posttranslational modification of a protein is crucial for the expression of its functional aspects and, therefore, it is under strict regulation. Whereas phosphorylation and dephosphorylation have been well recognized to determine the activity of proteins, *O*-linked glycosylation has been recently recognized to participate in the modification of an increasing number of transcription factors and nuclear proteins (9, 10). The fact that the gene structure of Elf-1 encodes for multiple motifs that have the potential to undergo phosphorylation and glycosylation suggests that such modifications should represent an integral part of the biochemistry and biology of the Elf-1 protein.

Therefore, we have hypothesized that one or multiple posttranslational modifications of Elf-1 are responsible for the large gap between the observed and predicted molecular mass. Our experiments show that both protein kinase C (PKC)-mediated phosphorylation and *O*-*N*-acetylglucosamine (GlcNAc) glycosylation are responsible for the formation of the 98-kDa form of Elf-1. We found that these modifications are important in determining the subcellular localization, metabolism, and DNA binding ability of Elf-1.

Materials and Methods

Lymphocyte isolation and cell culture

The isolation and purification of primary T lymphocytes and culture of Jurkat cells have been recently described (11, 12).

Plasmids

The expression plasmid of Elf-1 was a kind gift of Dr. J. Leiden (Harvard University, Cambridge, MA). The PKC expression plasmids were generously provided by Dr. G. Baier (University of Innsbruck, Innsbruck, Austria).

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[3] Abbreviations used in this paper: Ets, E 26 specific; PKC, protein kinase C; Rb, retinoblastoma; GlcNAc, *N*-acetylglucosamine; pcDNA, plasmid-encoding cDNA; VP16, virion polypeptide 16.

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Reagents

Okadaic acid and bacterial phage λ phosphatase was purchased from Calbiochem (La Jolla, CA) and New England Biolabs (Beverly, MA), respectively. Kinase inhibitors PD98059, SB203580, and KT5720 were purchased from Calbiochem. Proteasome inhibitor LLnL (*N*-Ac-Leu-Leu-norleucinal) is a product of Sigma-Aldrich (St. Louis, MO). The anti-Elf-1 Ab was a kind gift of Dr. J. Leiden. Anti-Elf-1 Ab was also purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The other Abs were purchased from various manufacturers as follows: *O*-GlcNAc (RL-2) Ab from Affinity BioReagents (Golden, CO), PKC-θ Ab from BD Transduction Laboratories (Franklin Lakes, NJ), actin and Fli-1 Abs from Santa Cruz Biotechnology, phosphoserine Ab from Sigma-Aldrich, retinoblastoma (Rb) Ab from BD PharMingen (San Diego, CA), and *p*-CREB Ab from Upstate Biotechnology (Lake Placid, NY).

Protein purification and Western blotting

To prepare cytoplasmic extracts, cells (5×10^6) were incubated on ice in 400 μl of lysis buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA supplemented with freshly added 1 mM DTT, 0.5 mM PMSF, 2 mM aprotinin, 1 mM of leupeptin, 10 mM NaF, and 2 mM Na_3VO_4) for 15 min. At the end of the incubation, Nonidet P-40 was added to the reaction mixture at a concentration of 0.6%. The reaction mixture was then subjected to centrifugation at 13,000 rpm for 1 min, and the supernatant was saved as cytoplasmic extract. The pellet was first washed once with buffer A to remove the residual cytoplasmic proteins, then resuspended in 40 μl of buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA supplemented with 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 mM aprotinin, and 1 mM of leupeptin) and vortexed in the cold room for 20 min. The supernatant was used as nuclear extract. To prepare whole cellular extract, cells were lysed in buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40 and supplemented with 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 mM aprotinin, and 1 mM of leupeptin on ice for 30 min. After centrifugation, the supernatant was saved as whole cellular extract. Western blotting was performed by following the manufacturer's instruction (ECL; Amersham, Piscataway, NJ).

Transfection of primary human lymphocytes

The electroporation methods used for the transfection of Jurkat and primary T lymphocytes recently have been described (11–14).

Immunoprecipitation

Nuclear proteins (150 μg) were precleaned in the Kyriaki's buffer (20 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 1%Triton X-100, 10% glycerol supplemented with 1 mM Na_3VO_4,1 mM PMSF, 2 mM aprotinin, and 1 mM leupeptin) and 20 μl of protein A agarose beads at 4°C for 1 h. The reaction mixture was spun, and the supernatant was incubated with the protein agarose beads and specific Ab for another hour. The resulting beads were thoroughly washed with the same buffer five times. The beads were then resuspended in the SDS gel-loading buffer and boiled for 3 min, and the supernatant was subjected to SDS-gel electrophoresis and Western blotting.

EMSAs

Nuclear extracts (4 μg) were incubated with a radiolabeled probe (5′-TCGAGAACCTCCAGGGCTTCCTGCCTGTGAACCA-3′) and 1 μg of poly(dI:dC) in the binding buffer (20 mM Tris-HCl (pH 7.5), 50 mM KCl, 2.5 mM DTT, 0.5 mM EDTA, and 7.5% glycerol) for 15 min at room temperature. The reaction mixture was then subjected to separation in 6% nondenaturing gel (Invitrogen, Carlsbad, CA). The dried gel was then autoradiographed. For supershift assays, the nuclear proteins were incubated with specific Abs at room temperature for 30 min before the probe, poly(dI:dC), and binding buffer were added. The reaction was further conducted for another 15 min at room temperature.

Results

The 80-kDa Elf-1 resides mainly in the cytoplasm, whereas the 98-kDa Elf-1 is primarily in the nucleus of T cells

We first transfected primary human T and Jurkat cells, using a cell electroporation technique (11–14), either with the plasmid-encoding cDNA (pcDNA)/Elf-1 plasmid, which encodes the full-length Elf-1 cDNA, or with the empty plasmid to establish the protein products of the Elf-1 gene. The cytoplasmic extracts from primary (Fig. 1*a*) and Jurkat T cells (data not shown) transfected with the empty plasmid expressed the 80-kDa protein and barely any detectable levels of the 98-kDa form of Elf-1. Transfection of T cells with the pcDNA/Elf-1 vector resulted in enhanced expression of both 80- and 98-kDa bands, and the intensity of the 80-kDa band was 10 times higher than that of the 98-kDa band. These data suggest that both the 80- and 98-kDa forms of Elf-1 are directly related to the plasmid-encoded protein and that they do not represent products of differential mRNA splicing or other proteins homologous to Elf-1, which may have been recognized by the Ab used.

Next, we determined the subcellular localization of the different forms of Elf-1. Using cytoplasmic and nuclear extracts from primary and Jurkat T cells, we found that the 80-kDa form of Elf-1 was prevalent in the cytoplasmic extracts, whereas the 98-kDa form of Elf-1 was prevalent in the nuclear extracts (Fig. 1*b*). To ensure the purity of the cytoplasmic and nuclear preparations, we probed cytoplasmic and nuclear proteins from either Jurkat or primary T cells sequentially with Abs against *p*-CREB and PKC-θ, two proteins known to reside in the nuclear and cytoplasmic compartments, respectively. As shown in Fig. 1*b*, PKC-θ colocalized with the 80-kDa Elf-1 in the cytoplasm, whereas *p*-CREB did so with the 98-kDa form of Elf-1 in the nucleus. These data proved that the preparations of cytoplasmic and nuclear proteins were not cross-contaminated and that the 80- and 98-kDa forms of the Elf-1 displayed differential subcellular compartment localization.

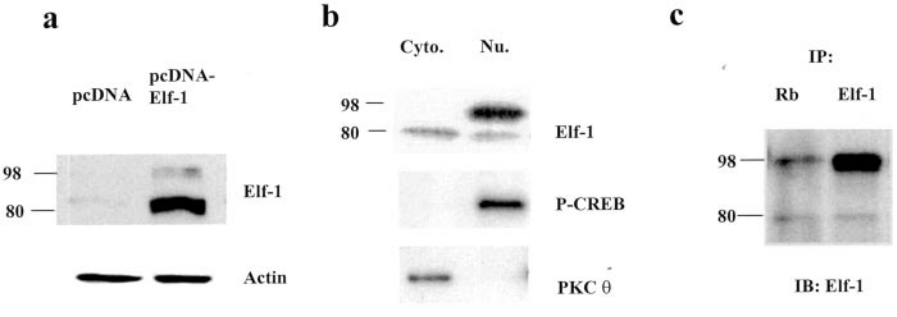

FIGURE 1. The 80-kDa form of Elf-1 resides in the cytoplasm, whereas the 98-kDa form of Elf-1 resides in the nucleus of T cells. *a*, Cytoplasmic proteins from primary human lymphocytes, transfected with control plasmid or pcDNA/Elf-1, were probed with Abs against Elf-1 or actin. *b*, Cytoplasmic and nuclear proteins from primary T cells were probed sequentially with Abs against Elf-1, phosphorylated CREB, and PKC-θ. *c*, Nuclear proteins from Jurkat cells were immunoprecipitated with Abs against either Elf-1 or Rb protein. The immunoprecipitated proteins were separated electrophoretically and then probed with an Ab against Elf-1.

It has been reported that the Rb protein retains Elf-1 in the cytoplasm and that, upon its phosphorylation, Elf-1 is free to translocate to the nucleus (15). To determine which form of Elf-1 binds to Rb, we immunoprecipitated nuclear extracts from T cells with Abs against the Rb and Elf-1 proteins. The anti-Elf-1 Ab immunoprecipitated both the 98- and 80-kDa bands (Fig. 1*c*) at a ratio (30:1) comparable to the input of nuclear proteins (see Fig. 1*b*). In contrast, the anti-Rb Ab immunoprecipitated 98- and 80-kDa Elf-1 at a ratio of 3:1, which is lower than that of the input of nuclear proteins, suggesting that Rb interacts better with the 80-kDa form than with the 98-kDa form. Therefore, the conversion of Elf-1 from its 80-kDa form to the 98-kDa form decreases its interaction with Rb and contributes to its nuclear migration.

PKC is involved in the phosphorylation of Elf-1

Because PKC-θ recently has been shown to be involved in the phosphorylation of several transcription factors involved in the regulation of the expression of T lymphocyte genes such as AP-1 (16), NF-κB (17), and CREB (12), we considered that similar PKC isoforms may also be involved in the phosphorylation of Elf-1 and may be responsible for the increase in the apparent molecular mass of Elf-1. In addition, sequence analysis of Elf-1, using the Expert Protein Analysis System proteomics server from the Swiss Institute of Bioinformatics, revealed the existence of multiple PKC phosphorylation sites. As shown in Fig. 2*a*, Jurkat cells cultured in the presence of PMA and calphostin C, which inhibits all the activities of PKC isoforms, displayed diminished expression of 98-kDa Elf-1. Similar results were obtained in the absence of PMA (see below). The PKC-θ inhibitor rotterlin also decreased the expression of 98-kDa Elf-1, although not to the same extent as calphostin C, suggesting that more than one isoform of PKC is involved in the conversion of the 80-kDa to the 98-kDa form. Interestingly, treatment of Jurkat cells with calphostin C enhanced the expression of 80-kDa Elf-1, indicating that the 98-kDa form can be derived directly from the 80-kDa form. Incubation of Jurkat cells with inhibitors of other kinases, such as PD98059 (mitogen-activated protein kinase), SB203580 (P38; stress-activated protein kinase), and KT5720 (protein kinase A), did not alter the expression of either the 98- or 80-kDa forms of Elf-1 (Fig. 2*a*). Furthermore, calphostin displays similar effects on the conversion of Elf-1 from 98- to 80-kDa form in the presence (Fig. 2*a*) or absence of PMA (see below).

To test directly which of the various PKC isoforms are involved in the phosphorylation of Elf-1, we transiently transfected Jurkat cells with a series of plasmids expressing different active PKC isoforms. As shown in Fig. 2*b*, Jurkat cells transfected with the empty vector expressed only the 80-kDa Elf-1 in the cytoplasmic extracts. Transient expression of either PKC-θ or PKC-ϵ increased the expression of the 98-kDa form, whereas overexpression of PKC-α did not affect the relative levels of 80- and 98-kDa forms of Elf-1. These data show that the so-called novel PKC isoforms θ and ϵ, but not the conventional PKC isoform α, are involved in the posttranslational modification of Elf-1.

The fact that the levels of 98-kDa Elf-1 increase after overexpression of PKC suggests that PKC-mediated phosphorylation is involved in the formation of the 98-kDa form of Elf-1. To confirm this hypothesis, we treated Jurkat T cells with different doses of okadaic acid, an inhibitor of Ser and Thr phosphatases. To determine whether phosphorylation of multiple PKC Elf-1 phosphorylation sites occurs gradually, we separated the proteins in 6% instead of 10% SDS gels (Fig. 2*c*). The 98-kDa Elf-1 resolved into three bands, and the 80-kDa Elf-1 resolved into two bands. Treatment of the cells with okadaic acid enhanced the expression of all three 98-kDa bands in a dose-dependent (100–500 nM) manner,

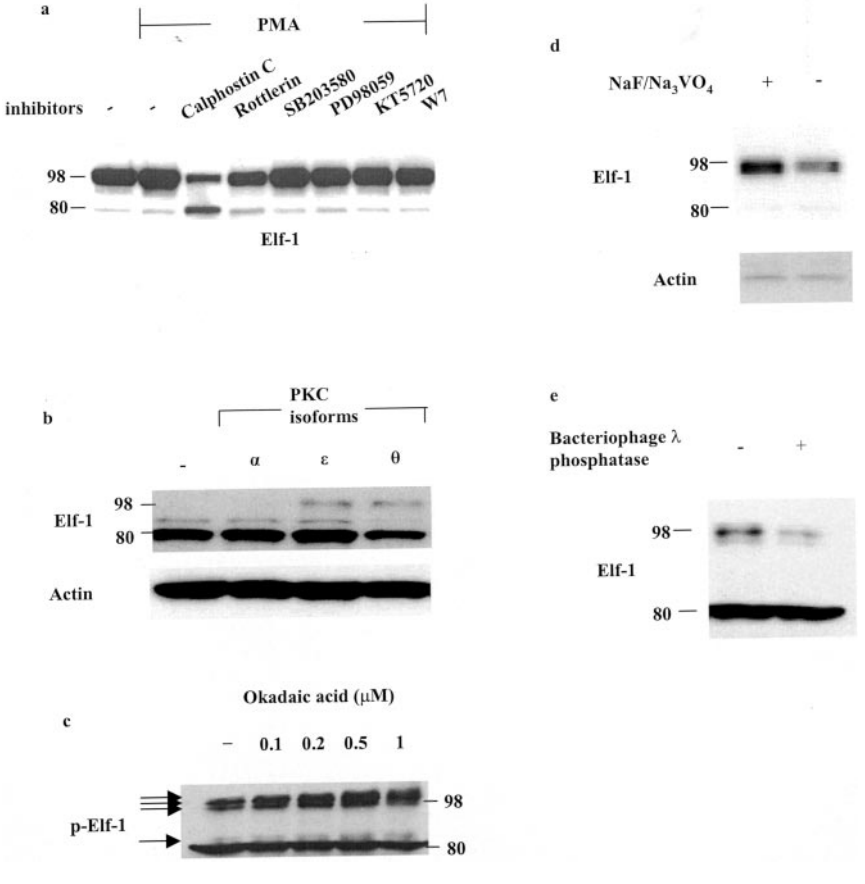

FIGURE 2. PKC-θ is involved in the conversion of Elf-1 from the 80- to the 98-kDa form. *a*, Primary T cells were treated for 30 min with various kinase inhibitors: calphostin C (0.05 μM), rotterlin (30 μM), PD98059 (50 μM), SB203580 (10 μM), KT5720 (5 μM), or W7 (15 μM). They were then stimulated with PMA (10 ng/ml) for another 6 h. Nuclear proteins were then isolated, analyzed in SDS gels, and probed with an Ab against Elf-1. *b*, Cytoplasmic proteins from Jurkat cells (5×10^6) transiently transfected with 3 μg of either control vector or plasmids expressing constitutively active forms of PKC α, ϵ, θ were probed with Abs against Elf-1 and actin. *c*, Total cellular proteins from Jurkat cells treated with okadaic acid were separated by electrophoresis in 6% SDS polyacrylamide gels and probed with an Ab against Elf-1. Arrows indicate phosphorylated forms of Elf-1. *d*, Nuclear proteins were purified as described in *Materials and Methods*, except that when indicated the phosphatase inhibitors NaF and Na_3VO4 were not included in the extraction buffer. The resulting proteins were analyzed in SDS gel followed by Western blot with Abs against Elf-1 or actin. *e*, Total cellular proteins digested with bacteriophage λ phosphatase at 37°C overnight were analyzed in 6% SDS gel and probed with an Ab against Elf-1.

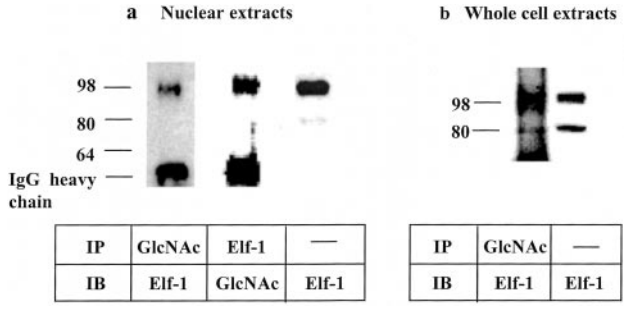

FIGURE 3. The 98- and 80-kDa forms of Elf-1 are *O*-GlcNAcylated. *a*, Nuclear proteins from Jurkat cells were immunoprecipitated with Abs against either *O*-GlcNAc (RL-2) or Elf-1 and were probed with Abs against Elf-1 or RL-2, respectively. *b*, Whole cell extracts were subjected to immunoprecipitation and immunoblotting as in *a*.

whereas it minimally enhanced the intensity of the upper 80-kDa Elf-1 band. These data indicate that in T cells cultured in FCS, in the absence of other exogenous stimuli, the 98-kDa form and only a small portion of the 80-kDa form of Elf-1 are phosphorylated.

To further investigate the role of phosphorylation in the expression of the Elf-1 forms, we performed two additional sets of experiments. First, we isolated cell lysates in the presence or absence of the phosphatase inhibitors NaF and Na_3VO_4, and we noted that the absence of these inhibitors results in decreased expression of the 98-kDa but not the 80-kDa Elf-1 form (Fig. 2*d*). When the cellular extracts, purified in the absence of phosphatase inhibitors, were incubated at 37°C for 18 h in the absence of phosphatase inhibitors, the 98-kDa form almost disappeared, whereas in the presence of the inhibitors, its disappearance was prevented (data not shown). Second, we treated total cellular proteins with the bacteriophage λ phosphatase. The bands that displayed enhanced intensity after treatment with okadaic acid decreased in intensity after treatment with the bacteriophage λ phosphatase (Fig. 2*e*). Altogether, these experiments indicate that the 98-kDa Elf-1 is heavily phosphorylated and undergoes dephosphorylation in a dynamic manner.

The 80- and 98-kDa forms of Elf-1 are O*-GlcNAc glycosylated*

Because the okadaic acid-induced hyperphosphorylation of Elf-1 (Fig. 2*c*) cannot account for the shift in the migration of Elf-1, we considered that glycosylation contributes to the appearance of the 98-kDa form of Elf-1. Elf-1 has multiple potential sites for *O*-GlcNAc glycosylation, and therefore we investigated whether Elf-1 is also *O*-GlcNAc modified. We first immunoprecipitated nuclear proteins either with an Ab against Elf-1 or with an Ab (RL-2) against the *O*-GlcNAc moiety (15, 18, 19) and then blotted the separated immunoprecipitates with the reverse Ab. Both processes revealed a 98-kDa band that is *O*-GlcNAc glycosylated (Fig. 3*a*). Because the nuclear proteins contain mainly the 98-kDa form and a barely detectable 80-kDa form, we repeated the above immunoprecipitation experiments using whole cell extracts. The whole cellular extract contains comparable amounts of both 98- and 80-kDa forms and therefore will help to further determine whether the 80-kDa Elf-1 is also glycosylated. Fig. 3*b* shows that the 80-kDa form is also recognized by the RL-2 Ab, and therefore it is also decorated with GlcNAc moieties. However, the interaction between RL-2 Ab and the 98-kDa form is more than eight times stronger than that between RL-2 and the 80-kDa form, as determined by densitometry. These experiments indicate that the interactions among the different forms of Elf-1 and RL-2 are specifically related to the extent of glycosylation and indicate that the glycosylation contributes to the difference in the apparent molecular mass between the 98- and 80-kDa forms.

The Elf-1 that binds to the TCR ζ-chain promoter is both glycosylated and phosphorylated

To determine the functional consequences of both the phosphorylation and glycosylation on Elf-1, we performed EMSA analysis using nuclear proteins from primary and Jurkat T cells and a labeled oligonucleotide spanning the Elf-1 binding site (−147/−119) of the TCR ζ-chain promoter. We observed two major bands resulting from the binding of nuclear proteins to the Elf-1 oligonucleotide (Fig. 4*a*). The upper band represents specific binding because its intensity decreases dramatically in the presence of an anti-Elf-1 Ab. In contrast, the incubation of nuclear proteins

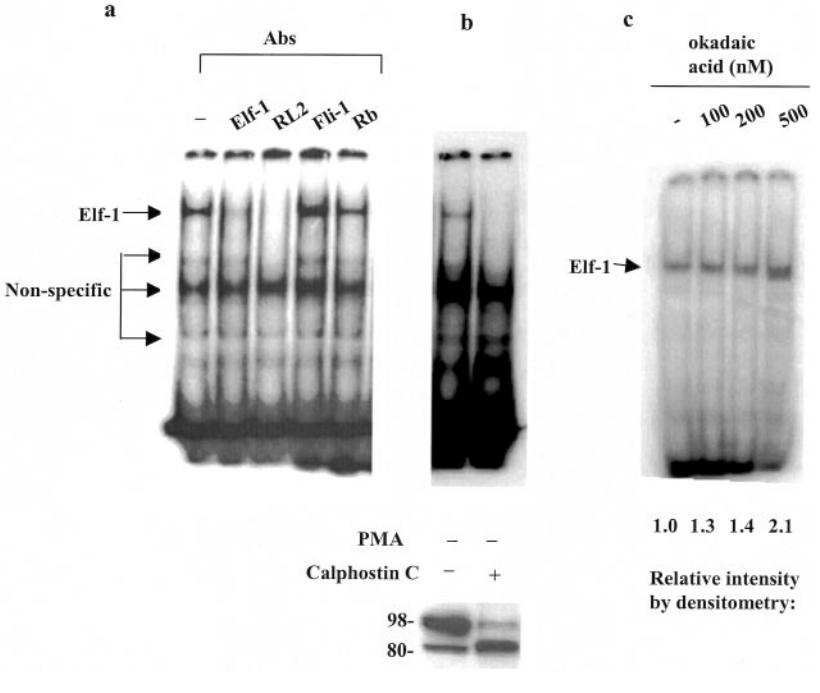

FIGURE 4. The Elf-1 that binds to TCR ζ-chain promoter is both phosphorylated and glycosylated. *a*, Nuclear proteins from Jurkat cells were incubated with a labeled oligonucleotide (−147/−119; TCR ζ-chain promoter) and Abs against Elf-1, RL-2 (anti-*O*-GlcNAc Ab), Fli-1, and Rb. The resulting DNA-protein complexes were analyzed by EMSA. *b*, Nuclear proteins from cells treated with and without calphostin C (*lower panel*) were incubated with the TCR ζ-chain-defined Elf-1 site; the resulting DNA-protein complex was resolved by EMSA. *c*, Total cellular proteins from Jurkat cells treated with okadaic acid (100–500 nM) were dialyzed to remove the SDS and Nonidet P-40 that were used to lyse the cells, incubated with the same Elf-1-binding oligonucleotide, and analyzed by EMSA. The numbers *below* indicate the intensity of the Elf-1/DNA binding complex as measured by densitometry.

with Abs against Fli-1 (a member of the Ets family) or Rb did not affect the binding patterns. Significantly, the presence of the anti-*O*-GlcNAc Ab, RL-2, in the EMSA reaction completely prevented the formation of the upper band, providing additional evidence that the Elf-1 protein that binds to the TCR ζ-chain promoter contains *O*-GlcNAc moieties (Fig. 4*a*).

Next, we conducted EMSA experiments by using nuclear proteins from cells treated with calphostin C, which inhibits the PKC-mediated modification of Elf-1 and results in increased amounts of 80-kDa Elf-1 and decreased amounts of 98-kDa Elf-1 (Fig. 4*b*, *lower panel*). Pretreatment of cells with calphostin C decreased Elf-1 binding (Fig. 4*b*). To further prove that the 98-kDa Elf-1 represents a phosphorylated form of Elf-1, we incubated nuclear extracts from Jurkat cells with an anti-phosphoserine Ab. This Ab prevented the formation of the upper band, but it did not affect the lower band (data not shown). Also, we dialyzed the whole cellular extracts from cells treated with okadaic acid (Fig. 2*c*) to remove the detergent and incubated them with the Elf-1 oligonucleotide. Okadaic acid enhanced the binding of the cellular proteins to the ζ-chain promoter-defined Elf-1 site in a dose-dependent manner (Fig. 4*c*). Taken together, these data indicate that both glycosylation and phosphorylation are required for Elf-1 to bind the Elf-1 site on the TCR ζ-chain promoter.

The expression levels of the 98-kDa form, but not the 80-kDa form, of Elf-1 in T cells correlate with the binding to the Elf-1 site on the TCR ζ-chain promoter

We showed above (Fig. 4*b*) that the nuclear proteins from cells treated with calphostin, which contains more 80-kDa Elf-1 and less 98-kDa Elf-1, display decreased DNA binding to the Elf-1 site on the TCR ζ-chain promoter. This observation supported the notion that the 98-kDa form of Elf-1 is the one mainly responsible for its DNA binding. Because we observed that samples from various donors differ between themselves in the expression level of the 80- and 98-kDa Elf-1, we analyzed samples in parallel by immunoblotting, using an anti-Elf-1 Ab, and with EMSA using a labeled Elf-1 oligonucleotide. Fig. 5*a* demonstrates that the nuclear proteins from individual blood donors have unique expression patterns of 98/80-kDa forms of Elf-1. In Fig. 5*b* the same samples were analyzed by EMSA to determine their ability to bind to DNA. Specificity of the binding was established by using anti-Elf-1 or control anti-Fli-1 Ab as discussed in Fig. 4*a*. Fig. 5, *a* and *b*, shows that the intensity of the specific DNA binding bands in different donors reflects the expression levels of the 98-kDa form, but not the 80-kDa form. Differences in binding of nuclear proteins to the Elf-1 site of the TCR ζ-chain promoter are not mirrored in the binding of the same nuclear proteins to other oligonucleotides, such as the one defined by the IL-2 promoter (−164/−189) (11) (Fig. 5*c*).

The 98-kDa Elf-1, but not the 80-kDa Elf-1, is degraded through the proteasome pathway

Elf-1 is present in the nucleus in its 98-kDa form, which is phosphorylated and able to bind to the promoter of the TCR ζ-chain. We considered that the phosphorylated 98-kDa Elf-1 undergoes degradation to prevent excessive accumulation. Because many transcription factors are degraded through the proteasome pathway, we treated Jurkat cells with different doses of the proteasome inhibitor LLnL for variable time periods. As shown in Fig. 6*a*, in the presence of LLnL for 3 h, the cytoplasmic levels of the 98-kDa Elf-1 increased significantly, indicating that the 98-kDa Elf-1 is constantly degraded through the proteasome pathway. In contrast, the 80-kDa Elf-1 did not change in the presence of LLnL, suggesting that this form is not degraded by the proteasome pathway. Compared with the cytoplasmic 98-kDa Elf-1, the nuclear 98-kDa Elf-1 did not increase rapidly after treatment with LLnL. Instead, it slowly decreased over time (Fig. 6*b*).

Discussion

We have found that PKC plays an active role in the conversion of 80-kDa form of Elf-1 to its 98-kDa form. T cells treated with the general PKC inhibitor calphostin C significantly decrease the level of the 98-kDa form, with the reciprocal increase of the 80-kDa form. Treatment of T cells with rotterlin, a PKC-θ-specific inhibitor, also had an effect similar to that of calphostin C. The fact that calphostin C has a more prominent effect than that of rotterlin

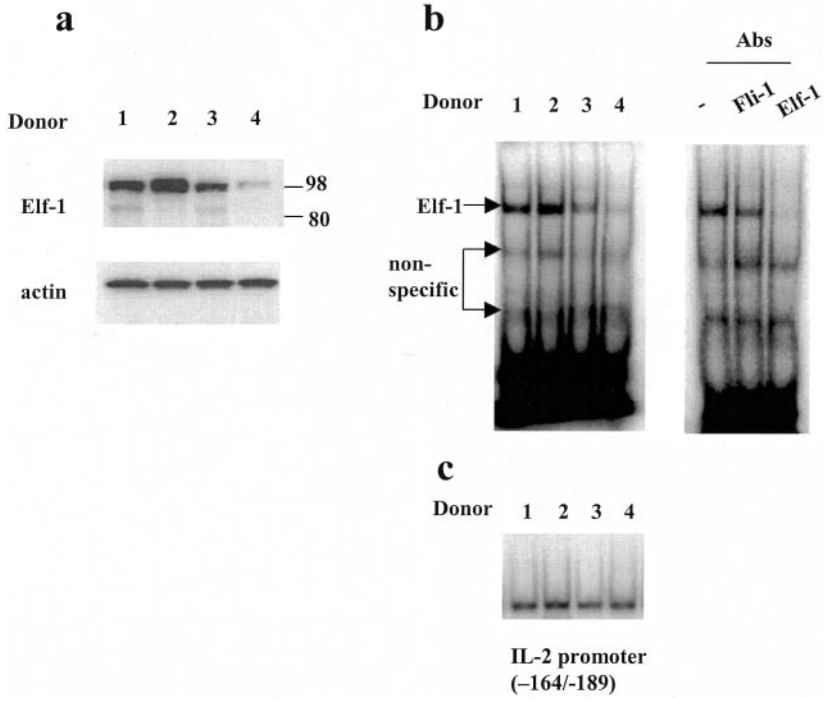

FIGURE 5. The expression level of the 98-kDa form but not the 80-kDa form of Elf-1 from different blood donors correlated with their DNA binding activity. *a*, Nuclear proteins were fractionated in the SDS gel and blotted with Abs against Elf-1 and actin. *b*, The nuclear proteins used in *a* were incubated with the oligonucleotides encoding the Elf-1 binding site on the TCR ζ-chain promoter followed by analysis with EMSA in the absence or presence of Abs against Elf-1 and Fli-1. *c*, As the loading control, the same nuclear proteins were incubated with an oligonulceotide encoding an IL-2 promoter-defined oligonucleotide (−164/−189) followed by analysis with EMSA.

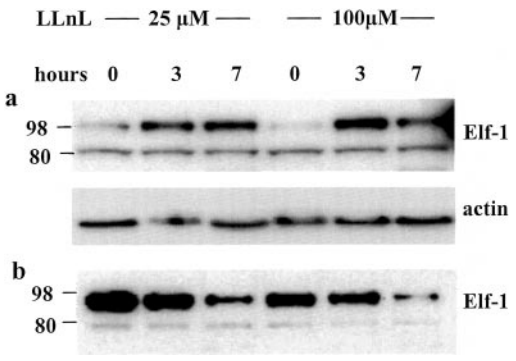

FIGURE 6. The cytoplasmic 98-kDa form but not the 80-kDa form of Elf-1 is degraded through the proteasome pathway. Jurkat cells were treated with either vehicle (DMSO) or different concentrations of the proteasome inhibitor LLnL for different time periods as indicated. Cells were harvested and cytoplasmic proteins (*a*) or nuclear proteins (*b*) were blotted with Ab against Elf-1.

indicates that more than one class of PKC is involved in the conversion of Elf-1 from 80 to 98 kDa. Furthermore, transfection of PKC-θ (a member of the novel PKC subfamily) into T lymphocytes resulted in the increase of the 98-kDa form at the cost of the 80-kDa form (Fig. 2*b*). In contrast, we were unable to demonstrate that transfection of PKC-α (a member of the conventional PKC subfamily) can change the ratio between these two forms. The other class of PKC, which is able to convert Elf-1 from 80- to 98-kDa form, may contain members of the atypical subfamily, such as PKC-ζ. Finally, sequence analysis discloses that Elf-1 encodes >10 potential PKC sites.

To confirm that the conversion of Elf-1 from 80- to 98-kDa form is accompanied by the phosphorylation of Elf-1, we have treated T lymphocytes with okadaic acid. This phosphatase inhibitor significantly increased the level of the 98-kDa form, but only minimally increased that of the 80-kDa form (Fig. 2*c*). This finding indicates that compared with the 80-kDa form, the 98-kDa form is more susceptible to a phosphatase-regulated process. We have further digested the total cellular proteins with bacteriophage λ phosphatase. Although the 98-kDa form was extensively digested, the 80-kDa form of Elf-1 was not affected (Fig. 2*e*). It is interesting that the decrease in the intensity of the 98-kDa form is not associated with an increase in the intensity of the 80-kDa form. This apparently means that dephosphorylation triggers the degradation of the 98-kDa form of Elf-1 rather than its return to the 80-kDa form. The same mechanism has been implicated in the degradation of activating transcription factor-2 (20) and the protooncogene Mos (21).

Altogether, these data indicate that the PKC-mediated conversion of Elf-1 from 80- to 98-kDa form involves the phosphorylation of Elf-1. It has not been determined whether PKC phosphorylates Elf-1 protein directly or indirectly. In other data (not shown), we found that transfection of an active form of Raf into T cells also causes phosphorylation of Elf-1. Because Raf can be activated by PKC (22), it is possible that the PKC may be involved in the phosphorylation of Elf-1 indirectly.

In addition to phosphorylation, we have demonstrated that glycosylation is also responsible in part for the increment in the apparent molecular mass of Elf-1. Whereas *N*-linked glycosylation frequently occurs in cell surface and secreted proteins, *O*-linked glycosylation, specifically the addition of *O*-GlcNAc to the hydroxyl groups on the side chains of Ser and Thr, represents the glycosylated form of an increasing number of transcription factors, such as Sp1, AP-1, AP-2, Hnf-1, c-Myc, p53, v-Erb-A, estrogen receptor, serum response factor (the protein regulating both gene expression and DNA replication (SV40 large T Ag)), the nuclear pore proteins, RNA polymerase II, and various chromatin proteins (9, 10). The results from the immunoprecipitation (Fig. 3*a*) and the gel shift assays (Fig. 4*a*) prove that Elf-1 undergoes *O*-GlcNAc modification. The number of GlcNAc moieties attached to each Elf-1 molecule is currently under investigation in our laboratory. Computer analysis using the Expert Protein Analysis System proteomics server from the Swiss Institute of Bioinformatics has indicated the presence of at least 20 residues on the Elf-1 (11 Thr and 9 Ser) that have high possibility (0.75–0.98, which is above the threshold) for *O*-GlcNAc modification. When total T cell extracts, which contain comparable amounts of 98- and 80-kDa Elf-1, were used for immunoprecipitation with the RL-2 Ab (Fig. 3*b*), the immunoprecipitates comprise mainly the 98-kDa form of Elf-1 (98-kDa form:80-kDa form = 8:1, as determined by densitometry). This finding indicates that 98-kDa Elf-1 is highly *O*-GlcNAc modified, which results in the strong interaction between RL-2 Ab and Elf-1. Phosphorylation and glycosylation have been shown to coexist in numerous transcription factors (9, 10). The exact relation and hierarchy, inasmuch as they concern the addition of phospho or glycosyl groups to the Elf-1 protein, are not clear at this point.

We have found that the conversion of Elf-1 from the 80-kDa to the 98-kDa form has multiple functional consequences. First, we have found that the 98-kDa form has less affinity to its cytoplasmic tethering protein Rb than the 80-kDa form (Fig. 1*c*). It is likely that the phosphoryl as well as the glycosyl groups block the access of Elf-1 to Rb. This mechanism, in conjunction with the phosphorylation of Rb, which was previously found to help releasing the bound Elf-1 (15), should be important in the control of the subcellular distribution of Elf-1. Second, by using EMSA, we found that the Elf-1 bound to the TCR ζ-chain promoter is glycosylated (Fig. 4*a*). Also, nuclear proteins from okadaic acid-treated T cells demonstrated increased DNA binding to the TCR ζ-chain promoter in a dose-dependent manner (Fig. 4*c*). Consistently, treatment of T cells with calphostin C, which should decrease the PKC-mediated phosphorylation, decreased this binding (Fig. 4*b*).

Although we have identified both phosphorylation and glycosylation to be involved in the conversion of Elf-1 from 80- to 98-kDa form, it is not known whether other posttranslational modifications are also involved. Considering the huge molecular mass discrepancy between these two forms (18 kDa), it is likely that additional modifications are involved. One potential mechanism that may be involved in the formation of the 98-kDa form of Elf-1 is ubiquitination. Uni- or di-ubiquitination can endow their modified proteins with an increase of 8 and 16 kDa, respectively, because ubiquitin is a peptide of 7.6 kDa. Accumulated evidence in recent years has essentially overthrown the notion that ubiquitination is a mechanism solely involved in targeting proteins for degradation. Ubiquitination has been found to play a role, beyond protein degradation, in receptor endocytosis, signaling transduction, cell cycle progression, and transcription regulation (23). Ligation of ubiquitin to the transactivation domain of transcription factor virion polypeptide 16 (VP16) is essential for its transaction capacity. VP16 loses its transaction capacity in yeast strains, which are defective in the expression of the VP16-responsible ubiquitin-ligase Met^{30} (24). However, an anti-ubiquitin Ab failed to detect any protein in nuclear lysates immunoprecipitated with an anti-Elf-1 Ab (data not shown), suggesting that ubiquitination is not involved in the posttranslational modification of Elf-1.

We and others (8) have reproducibly found that the 98-kDa form of Elf-1 exists abundantly in the nucleus in a variety of cell types. Our study further reveals that this form has multiple features that characterize active transcription factors: it resides in the nucleus, is highly regulated by phosphorylation, constitutively binds to DNA,

and is subject to tight control by the proteasome pathway. The maintenance of high level of this constitutively active form of transcription factor implies that Elf-1-dependent genes have essential functions and are indispensable.In contrast, it is mandatory for the cell homeostasis to tightly control this highly active transcription factor. We have demonstrated that the cytoplasm-located 98-kDa Elf-1 is accumulated rapidly in response to the inhibition of the proteasome pathway, whereas the same treatment resulted in the decrease of the 98-kDa form in the nucleus. LLnL did not promote the nuclear export of the 98-kDa form to the cytoplasm because the addition of leptomycin, a commonly used nuclear export inhibitor (25), failed to inhibit the decrease of the 98-kDa form in the nucleus (data not shown).

In conclusion, we found that in Jurkat and primary T cells, post-translational phosphorylation and glycosylation of Elf-1 are responsible for the observed two major 98- and 80-kDa forms. The 98-kDa form migrates to the nucleus, binds to DNA, and activates gene transcription. Our findings illustrate the mechanisms that govern the activation and the degradation of Elf-1 and should help the understanding of the pathogenesis of diseases associated with abnormal expression of Elf-1.

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