Calcium-Induced Phosphorylation of ETS1 Inhibits Its Specific DNA Binding Activity*

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Ets1, the founding member of the Ets gene family of transcriptional regulators, is a phosphoprotein which is highly expressed in cells of the T and B lymphoid lineages. Previous studies have shown that Ets1 becomes rapidly and transiently phosphorylated following antigen receptor (T cell (antigen) receptor (TCR) and membrane Ig) triggering a response which is absolutely dependent on ligand-induced calcium mobilization.

By a combination of two-dimensional tryptic phosphopeptide and mutational analyses, the target residues of these calcium-dependent phosphorylation events are identified as 4 serine residues clustered in a domain of Ets1 adjacent to its DNA binding domain (Ets domain). From the comparison of the properties of wild type Ets1 with those of mutant proteins carrying serine-to-alanine substitution in target residues, calcium-dependent phosphorylation of Ets1 is shown to inhibit its binding to specific DNA sequences but does not affect its ability to accumulate in the nucleus, another property dependent on the Ets domain.

Our data are consistent with a model in which the calcium-dependent phosphorylation of Ets1 represent the first step of a general clearance of Ets1 function during T and B cell activation.

Ets1 is the prototype of a gene family of about 20 members which encodes a novel class of sequence-specific transcriptional regulators involved in the response of cells to a variety of developmental and environmental cues (Bosselut et al., 1990; Gunther et al., 1990; Klemas et al., 1990; Wasylyk et al., 1990). Ets proteins share a region of similarity of about 85 amino acid residues, the Ets domain, which is sufficient for both nuclear accumulation and sequence-specific binding to DNA (Boulukos et al., 1989; Gunther et al., 1990; Gégonne et al., 1992; Lim et al., 1992). Ets response elements identified in a variety of promoters and enhancers, as well as Ets binding sites selected in vitro from random sequences, span about 10 nucleotides centered over a central GGAAT/T core sequence. In vitro, both sequences within the core, as well as sequences flanking it, contribute to DNA binding specificity as well as to DNA binding selectivity among different members of the family (Fisher et al., 1991a; Woods et al., 1992; Nye et al., 1992; Bosselut et al., 1993; Brown and McKnight, 1993).

Several lines of evidence implicate Ets family members in developmental processes and in the response of cells to a variety of extracellular signals. In Drosophila melanogaster, E74 is one of the early genes induced in larval tissues in response to ecdysone, and Yan/Pokkuri has been genetically implicated as a negative modulator of the differentiation of R7 photoreceptor precursor cells in response to the Sevenless signal transduction pathway (Burtis et al., 1990; Lai and Rubin, 1992; Tei et al., 1992). In higher eucaryotes, expression of several members of the Ets family is rapidly and transiently regulated in response to growth and differentiation stimuli (Bhat et al., 1987, 1990; Boulukos et al., 1990; Thummel et al., 1990; Seth et al., 1994), and Ets binding sites have been shown to contribute to the transcriptional response of a variety of promoters/enhancers to serum, phorbol esters, antigen, and non-nuclear oncogenes (Wasylyk et al., 1989; Prosser et al., 1992). Although in most of these cases the members of the family actually involved remain to be identified, recent evidence has shown that the growth factor receptor tyrosine kinase signaling pathway involving the Ras/Raf/microtubule-associated protein kinase cascade directly controls the activity of the Elk-1/SAP subfamily of Ets proteins (Marais et al., 1993; Hill et al., 1993). Furthermore, a dominant negative form of Ets2 is able to impair transformation by an oncogenic Ras gene as well as colony-stimulating factor type 1 mitogenic signaling in NIH3T3 cells expressing an ectopic colony-stimulating factor type 1 receptor (Langer et al., 1992). Finally, several members of the Ets family have been implicated in oncogenic processes. c-ets1 is the cellular progenitor of the v-ets oncogene of the avian leukemia virus E26 (Leprince et al., 1983; Nunn et al., 1983; Sipaly-Fu-Pu-1 and Fli-1 are found to be overexpressed in the leukemic cells of Friend virus-induced erythroleukemias as the result of nearby proviral insertions (Moreau-Gachelin et al., 1988; Ben-David et al., 1991); the DNA binding domain Fli-1 or ERG is fused to the 5′-half of the EWS gene in Ewing sarcoma and related tumors as the result of a specific t(11;22) chromosomal rearrangements (Delattre et al., 1992; Zucman et al., 1993).

Ets1 is widely expressed in early embryos, but becomes progressively tissue-restricted at later stages of development (Vandenbunder et al., 1989; Kola et al., 1993). In adults, expression is observed only in a few tissues, particularly lymphoid cells of the T and B cell lineages (Ghysdael et al., 1986). Antigenic stimulation of quiescent T and B lymphocytes results within minutes in the phosphorylation of Ets1 (Pogonose et al., 1988, 1990; Koizumi et al., 1990; Fisher et al., 1991b). These phosphorylation events are transient and depend upon the increase in the intracellular calcium ion concentration characteristic of T and B cell activation. In this manuscript, we identify the target residues involved in the calcium-dependent phospho-

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**MATERIALS AND METHODS**

**Plasmids Constructions**—All mutants described in this study were obtained by oligonucleotide site-directed mutagenesis using the single-stranded DNA of an M13mp7 chicken c-ets1 recombinant phage as substrate (Boulukos et al., 1989), using the Amberash kit.

Mutagenized inserts were recovered by EcoRI/HindIII digestion and subcloned in the same sites of the SV40-based Δ EB Ets1 expression vector (Boulukos et al., 1989). The following mutagenic oligonucleotides were used: Mut 1, 5'-CTACGTAGGAGGAGGCGGC-3'; Mut2, 5'-GGTACTGTCCTGTCGCTCCAAAGGGCATGCTAGGAA-3'; Mut3, 5'-GTTAGTCCTTCGTCAGTCCCAAGGCGATGCTGGGA-3'; Mut4, 5'-CTGTAGCCGTTCAGGGTGCC-3'; Mut5, oligonucleotide used for mut4 and mut1; Mut6, 5'-GGGAGCAGGCGCTGAGGCGGCTAGAAAGGAGGGCC-3'; Mut7, oligonucleotides used for mut4 and mut3; Mut8, 5'-CTTCCTGACTACGGGAGGACGCCTC-3'; Mut9, oligonucleotide used for mut8 and 5'-CTGGAGCAGCTGAGGCGCCTCAGGACGCGCGCC-3'. A Xhol-Sacl restriction fragment of Ets1 was subcloned in a derivative version of our Δ EB expression vector providing an in frame initiation codon followed by a series of serine residues clustered in a domain of the molecule adjacent to its DNA binding domain (Ets domain). Furthermore we show that these phosphorylation events inhibit the binding of Ets1 to specific DNA sequences but do not affect the ability of Ets1 to accumulate in the nucleus.

**Fig. 1.** Schematic structure of Ets1 deletion mutants and analysis of their phosphorylation in transfected COS cells. Panel A, schematic of wild type Ets1 and deletion mutants derived thereof. Ets-1A243-330 and Ets1 N319 have been described previously (Gegonne et al., 1992). The Ets (DNA binding) domain is shown as a black box, the domain encoded by c-ets1 exon 7 as a hatched box, and the transcriptional activation domain as a dense stippled box. The influenza hemagglutinin tag is also shown as a thinly stippled box. Panel B, COS-1 cells were transfected with expression vectors for Ets1 N244 or Ets 1 N319 using the DEAE-dextran method. [35S]Methionine/cysteine and [32P]orthophosphate labeling and ionomycin treatment were as in Fig. 1C. Immunoprecipitation was performed with the Ets specific antiserum.

**Fig. 2.** Stimulation of Ets1 and Ets1 Δ243-330 phosphorylation in transfected cells following treatment with ionomycin and anti-CD3 monoclonal antibodies. Panel A, Jurkat cells were transfected with expression vectors encoding hemagglutinin-epitope tagged Ets1 and Ets1 Δ243-330. Twenty-four hours after transfection, cells were labeled for 4 h with [32P]orthophosphate. Ionomycin (2 μM final concentration) or 0.3 μg/ml anti CD3 monoclonal antibody were added 5 min prior to cell lysis. Cell extracts (2 x 10⁶ acid-precipitable radioactive counts) were immunoprecipitated with the hemagglutinin epitope-specific 12CA5 monoclonal antibody, and immunoprecipitates were analyzed by electrophoresis on polyacrylamide gels followed by fluorography. Mock-transfected cells were used as control. Panel B, Western blot analyses of extracts (2 x 10⁶ cells/ lane) of parallel cultures of unlabelled transfected Jurkat cells using the 12CA5 monoclonal antibody. Two days after transfection cells were radiolabeled either with [32P]orthophosphate. Cells were stimulated with 2 μM ionomycin 5 min prior to cell lysis. Immunoprecipitation was performed using the Ets specific antiserum. Mock-transfected cells were used as control.

**Panel A**

- Ets1
- Ets1Δ243-330
- tag-Ets1
- Ets1Δ243-330
- tag-Ets1Δ243-330
- Ets1 N244
- Ets1 N319

**Panel B**

- 35S
- 32P
Analyses-COS-1 cells were maintained and transfected as described previously (Boulukos et al., 1989). Two days after transfection, cells were co-transfected by electroporation of 20 pg of SV40-derived vector and stimulated conditions.

The resulting sequence of the protein is as follows: YASLPSRAAVDLKP (bold letters, epitope; italic letters, Ets1).

Peptides not consistently detected in different experiments. Peptides 6 and 7 are often found to resolve into several species presumably as consequence of oxidation artifacts.

The tagged version of Ets1 Δ243–330 was obtained by replacing the EcoRVI HindIII fragment of the Ets1 A243–330 cDNA into the same vector used for making Ets1 319441 (see Gégonne et al. (1992)). These point mutations were chosen such that they do not alter the amino acid sequence of the protein. The resulting protein does not alter the amino acid sequence of the protein.

Ionomycin and anti-CD3 monoclonal antibody (clone UCHT, Serotec) were used as indicated in figure legends.

HeLa cells were maintained and transfected as described previously (Gégonne et al., 1993).

Two-dimensional Tryptic Phosphopeptide and Phosphoamino Acid Analyses and Electrophoretic Mobility Shift Assays—Procedures for two-dimensional tryptic phosphopeptide and phosphoamino acid analysis followed published procedures (Goldberg et al., 1988; Pognonec et al., 1988). Analysis of Ets1 binding made use of whole cell extracts of Jurkat cells transiently expressing Ets1. Cells were collected 1 day after transfection and lysed in 200 μl of 10 mM Hepes (pH 7.9), 0.15 M NaCl, 0.15 mM NaF, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Triton X-100, 1% aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM 2-mercaptoethanol, 1 mM β-glycerophosphate and centrifuged at 10,000 × g for 10 min. Binding reactions were performed with 4 μg of cell extract in a final volume of 16 μl for 10 min at 0 °C in the presence of nonspecific competitors (1.5 μg of poly(dI-dC), 0.4 μg of salmon sperm DNA), followed by the addition of 100 fmol of the 32P-labeled oligonucleotide probe for 10 min. The Ets1 oligonucleotide corresponds to an optimized high-affinity Ets1 binding site 5'-ATAAAACAG-GAAGTGTT-3' (Bosselut et al., 1993) flanked by AvaI restriction sites, to allow for radioactive labeling by filling in with the Klenow fragment of DNA polymerase I.

Immunofluorescence Studies—Jurkat cells transiently transfected with the SV40-based expression vector for the tagged version of Ets1 were transferred to microscope slides by cytocentrifugation (1000 × g, 10 min). Cells were washed twice in phosphate-buffered saline, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 during 2 min, and washed again with phosphate-buffered saline. Subcellular localization was analyzed by immunofluorescence with 12CA5 monoclonal antibody (1/1000 dilution of an ascites fluid) and anti-mouse fluorescein isothiocyanate-conjugated monoclonal antibody (Sigma).

1 R. A. Bailly and J. Ghysdael, unpublished data.
identified in T cells which failed to respond by increased phosphorylation to TCR engagement (Koizumi et al., 1990; Pognonec et al., 1990) and which was presumed to correspond to the translation product of an alternatively spliced mRNA lacking c-ets1 exon 7 (Reddy and Rao, 1988). We therefore analyzed the properties of an Ets1 protein, Ets1Δ243–330, in which amino acid residues 243–330, corresponding to the domain encoded by c-ets1 exon 7, are deleted. As shown in Fig. 1, the exogenously expressed Ets1Δ243–330 showed a reduced hyperphosphorylation upon T cell activation as compared with wild type Ets1 (Fig. 1, panel A), and unlike Ets1, phosphorylation of Ets1Δ243–330 was not accompanied by a change in electrophoretic mobility in SDS-polyacrylamide gels (Fig. 1, panel B).

Qualitatively similar results were obtained following ionomycin treatment of COS-1 cells transfected with Ets1 and Ets1Δ243–330 expression plasmids and labeled with either [32P]orthophosphate or a [35S]methionine/[35S]cysteine mixture (Fig. 1, panel C). This, together with the fact that the same phosphopeptides were identified in Ets1 isolated either from ionomycin-treated Jurkat or COS cells (Fig. 3, panels A and E), indicated that COS cells represent a suitable system to analyze in further details the calcium-dependent phosphorylation of Ets1.

Two-dimensional tryptic peptide analyses of phosphorylated Ets1 proteins revealed that ionomycin treatment of transfected COS cells resulted in the over-representation of four phosphopeptides (Fig. 3, compare panels A and B). These peptides will be referred to as peptides 1–4 (Fig. 3, panel F). Phosphoamino acid analysis showed that ionomycin treatment increased the phosphoserine to phosphothreonine ratio in Ets1, indicating that ionomycin-induced phosphorylation of Ets1 involved serine residues (Fig. 3, panel G).

The results of Fig. 1 showed that integrity of amino acid residues 243–330 is required for calcium-induced hyperphosphorylation and retarded electrophoretic mobility of Ets1. One interpretation of these results is that the target serine residues are all localized in this domain of Ets1. Alternatively, since this domain has been implicated in an intranuclear control of Ets1 DNA binding activity (Lim et al., 1992; Wasylyk et al., 1992), deletion of amino acid residues 243–330 could lead to a conformational change which in turn could affect phosphorylation of residues located elsewhere in the molecule. To address this issue, we made use of deletion mutants encoding either Ets1 residues 244–441 (Ets-1 N319) (see Fig. 2A for a schematic of these mutants). Both mutants share an intact Ets domain but differ by the presence of an entire exon 7-encoded domain in Ets1 N244. These mutants were found to be stably expressed in transfected COS cells (Fig. 2B, [35S]lanes) but only Ets1 N244 was found to be phosphorylated (Fig. 2B, [32P]lanes). Importantly, phosphorylation of Ets1 N244 was increased in response to ionomycin treatment (Fig. 2B), and increased phosphorylation occurred exclusively in phosphopeptides 1–4 as analyzed by two-dimensional tryptic peptide analysis (Fig. 3, compare panels C and D). These experiments indicated therefore that the calcium-dependent phosphorylation of Ets1 involves serine residues localized in the domain encoded by c-ets1 exon 7.

The relative migration of phosphopeptides 1 and 2, on the one hand, and phosphopeptides 3 and 4, on the other (i.e., two spots lying on a diagonal sloping toward the anode), is reminiscent of what can be expected from two phosphoisomers of the same peptide. Accordingly, phosphopeptides 1 and 3 would result from the addition of one additional phosphate group to phosphopeptides 2 and 4, respectively. Examination of the amino acid sequence encoded by c-ets1 exon 7 revealed that only three serine-containing tryptic peptides could be gener-
expressed in expressing Ets1 or mutants derived thereof were labeled with \[^{32}P\]orthophosphate and either left untreated (−) or treated for 5 min with 2 μM ionomycin (+). Ets1 proteins were immunoprecipitated with the Ets-specific antiserum and analyzed by electrophoresis on 10% polyacrylamide slab gels followed by autoradiography. Mock-transfected cells are used as control. Bottom gel lanes, Western blot analysis of Ets1 substitution mutants expressed in COS cells. An aliquot of the cells used in top gel lanes were processed for immunoblotting analyses. 2 x 10^6 cells of each lysate were electrophoresed on 10% polyacrylamide gels and transferred to nylon membrane. The membrane was incubated with the Ets-specific antiserum and revealed using the ECL (Amersham) chemiluminescence kit followed by autoradiography.

**Fig. 5. Analysis of the effect of ionomycin stimulation on the phosphorylation of Ets1 substitution mutants.** Top gel lanes, COS-1 cells expressing Ets1 or mutants derived thereof were labeled with \[^{32}P\]orthophosphate and either left untreated (−) or treated for 5 min with 2 μM ionomycin (+). Ets1 proteins were immunoprecipitated with the Ets-specific antiserum and analyzed by electrophoresis on 10% polyacrylamide slab gels followed by autoradiography. Bottom gel lanes, Western blot analysis of Ets1 substitution mutants expressed in COS cells. An aliquot of the cells used in top gel lanes were processed for immunoblotting analyses. 2 x 10^6 cells of each lysate were electrophoresed on 10% polyacrylamide gels and transferred to nylon membrane. The membrane was incubated with the Ets-specific antiserum and revealed using the ECL (Amersham) chemiluminescence kit followed by autoradiography.

The protein resulting from c-ets1 exon 7 (see Fig. 4) contains 4 serine residues separated by regularly spaced acidic (Glu or Asp) amino acids. Peptide II includes 6 serine residues embedded in a region rich in glutamine and hydrophobic residues. Peptide III contains 3 serine residues, one of them (Ser^{285}) being located in the consensus recognition sequence for calcium and calmodulin-dependent protein kinases (RXXS, Pearson et al., 1985). Of note, serine residue 285 is found in an amino acid sequence environment similar to that of serine 251 and 257 in peptide I, namely acidic-serine-aromatic-acidic.

We made use of these observations to mutate either singly or in combination the serine residues encoded by c-ets1 exon 7 (see Fig. 4 for a description of the mutants) and analyzed the effect of these mutations on the phosphorylation pattern and the electrophoretic mobility of Ets1-mut1 as compared with the wild type protein (Fig. 5). Two-dimensional tryptic phosphopeptide analysis of Ets1-mut1 showed the disappearance of phosphopeptide 1 (Fig. 6, compare panels A and C), demonstrating that Ser^{282} is a major phosphorylation site of Ets1. In line with our hypothesis that peptides 1 and 2 represent phosphoisomers of the same tryptic peptide, mutation of Ser^{282} and Ser^{285} to generate Ets1-mut2 or of Ser^{282} and Ser^{285} to generate Ets1-mut8 also resulted in an altered ionomycin-induced hyperphosphorylation and electrophoretic mobility of Ets1-mut1 as compared with the wild type protein (Fig. 5). Two-dimensional tryptic phosphopeptide analysis of Ets1-mut1 showed the disappearance of phosphopeptide 1 (Fig. 6, compare panels A and C), demonstrating that Ser^{282} is a major phosphorylation site of Ets1. In line with our hypothesis that peptides 1 and 2 represent phosphoisomers of the same tryptic peptide, mutation of Ser^{282} and Ser^{285} to generate Ets1-mut2 or of Ser^{282} and Ser^{285} to generate Ets1-mut8 also resulted in an altered ionomycin-induced hyperphosphorylation and electrophoretic mobility of Ets1 (Fig. 5) and in the disappearance of phosphopeptide 1 or both phosphopeptides 1 and 2, respectively (Fig. 6, compare panels A, F, and E). The protein resulting from the combined mutation of serine residues 282, 285, and 287 (Ets1-mut3) showed the same properties as Ets1-mut8 (data not shown). We conclude from these experiments that phosphopeptides 1 and 2 result from the presence of either one (phosphopeptide 2) or two phosphate groups (phosphopeptide 1) to Ser^{282} and/or Ser^{285} of peptide III.

Mutation of the 4 serine residues of peptide I into alanine to generate Ets-mut4 was accompanied by a modification of the phosphorylation and the electrophoretic mobility of the protein in response to ionomycin treatment (Fig. 5) and the specific disappearance of phosphopeptides 3 and 4 in two-dimensional mapping analyses (Fig. 6, panel B). Combination of the mutations of Ets1-mut4 with those of Ets1-mut3 to generate Ets1-mut7 or combination of the mutation of Ser^{282} and Ser^{285} with the mutations of Ets1-mut8 to generate Ets1-mut9 completely abolished the hyperphosphorylation and the reduced electrophoretic mobility of Ets1 normally observed in response to ionomycin (Fig. 5). These combined mutations also lead to the disappearance of phosphopeptides 1–4 as shown by two-dimensional phosphopeptide mapping analyses (Fig. 6, panel D and data not shown). Phosphopeptides 3 and 4 represent therefore phosphoisomers of peptide I corresponding to the addition of either one or two phosphate group to Ser^{282} and Ser^{285}. Consistent with the fact that all ionomycin-induced phosphopeptides in Ets1 are accounted for by phosphorylation of specific residues in peptides I and III, mutation of all serine residues of peptide II of Ets1-mut1 into alanine to generate Ets1-mut6 yielded a two-dimensional phosphopeptide pattern which is indistinguishable from that of Ets1-mut1 (Fig. 6, panels G and H).

We next analyzed the consequences of representative mutation in peptides I and III on the response of Ets1 to the stimulation of transfected Jurkat T cells by either ionomycin or an
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We previously reported that the calcium-dependent phosphorylation of Ets1 correlated with the inhibition of nonspecific binding of Ets1 to general DNA (Pognonec et al., 1989). We therefore analyzed whether the calcium-dependent phosphorylation events characterized here affected the ability of Ets1 to bind specific DNA sequences. The DNA binding activity of Ets1 was studied by electrophoretic mobility shift assays using as probe an oligonucleotide corresponding to a high affinity Ets1 binding site (oligonucleotide A; Bosselut et al., 1993) and whole cell extracts of Jurkat cells transfected either with an Ets1 expression plasmid or mock-transfected as control. The results of Fig. 8 show that overexpression of the epitope-tagged version of Ets1 in Jurkat cells resulted in the appearance of a novel retarded complex which migrated between two other complexes endogenous to these cells (Fig. 8, panel A, compare lanes 1 and 2). This novel complex corresponds to the binding of plasmid-encoded Ets1, since it is absent from extracts of control cells and since it is specifically supershifted by either an Ets1-specific antiserum or by the 12CA5 epitope-specific monoclonal antibody, but not by a control serum (Fig. 8, panel B). The Ets1-probe complex resulted from specific binding to the oligonucleotide A probe, since its formation was competed by a 100-fold molar excess of unlabeled oligonucleotide A, but not by the same excess of a mutated oligonucleotide A bearing a GG to CC transversion in the GGAA core sequence (Fig. 8, panel B). By the same criteria, the two complexes endogenous to Jurkat cells also represent specific binding to the probe and are likely to result from the specific binding of other Ets family members.

Treatment of Jurkat cells overexpressing Ets1 with ionomycin 5 min prior to preparation of whole cell extract, a treatment which induced quantitative conversion of Ets1 to its hyperphosphorylated form (Fig. 1), resulted in the specific inhibition of the formation of the Ets1-probe complex (Fig. 8, panel C, compare lanes 4 and 5), suggesting that Ets1 hyperphosphorylation abolished specific DNA binding activity. To analyze this point in further details, we compared the response of various Ets1 deletion and substitution mutants in this assay. Overexpression of mutant proteins bearing serine to alanine substitutions which affected only partially the calcium-induced phos-
perphosphorylation such as Ets1-mut1, Ets1-mut2, or Ets1-mut5 yielded specific probe-protein complexes the formation of which was only partially inhibited by ionomycin treatment (Fig. 8, panel C, lanes 8–13). In contrast, overexpression of Ets1-mut9, a form of Ets1 which failed to become hyperphosphorylated on any of the exon 7-encoded serine residues in response to ionomycin, yielded a specific probe-DNA complex which was unaffected by ionomycin treatment of transfected cells (Fig. 8, panel C, compare lanes 14 and 15). Overexpression of Ets1 Δ243–330, which lacked the entire exon 7-encoded domain, yielded a specific complex of slightly faster electrophoretic mobility than that generated by wild type Ets1 the formation of which was also unresponsive to treatment of cells with ionomycin (Fig. 8, panel C, compare lanes 6 and 7). We conclude from these experiments that phosphorylation of all target serine residues in the exon 7-encoded domain of Ets1 is required to inhibit the ability of the protein to bind specific DNA sequences.

Besides specific binding to DNA, the Ets domain of Ets1 also encodes the ability of the protein to localize to the nucleus (Boulukos et al., 1990). We therefore analyzed whether ionomycin treatment of transfected cells overexpressing Ets1 affected its nuclear localization. The results of Fig. 9 show that exogenous expression of Ets1 in either HeLa (Fig. 9, panel A) or Jurkat cells (Fig. 9, panel B) resulted in its accumulation in the nucleus of untreated cells as observed by immunofluorescence analysis (Fig. 9). Treatment of cells for various periods of time with ionomycin prior to fixation, although it resulted in the rapid and quantitative modification of Ets1 by phosphorylation as evidenced by its retarded electrophoretic mobility (Fig. 9, panel A), failed to affect the nuclear localization of the protein (Fig. 9, panels A and B) in either HeLa or Jurkat cells. We conclude from these experiments that the calcium-induced phosphorylation of Ets1 does not affect its ability to localize to the nucleus.

**DISCUSSION**

The results described in this study identify the major targets of the calcium-dependent phosphorylation events of Ets1 as 4 serine residues localized in a domain encoded by c-ets1 exon 7 which is adjacent to its DNA binding domain. Comparison of the properties of wild type Ets1 with those of mutant versions carrying serine-to-alanine substitutions of target serine residues in a T cell line, where calcium-dependent phosphorylation of Ets1 occurs in response to physiologic stimuli such as T cell (antigen) receptor engagement, show that these phosphorylation events inhibit the ability of Ets1 to bind specific DNA sequences.
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Two classes of target serine residues were identified. The first, Ser^{282}, is localized in a primary sequence environment which conforms to the consensus sequence for phosphorylation by calcium and calmodulin-dependent protein kinases (CaM kinases). A peptide corresponding to Ets1 exon 7 has been reported to be phosphorylated in vitro by CaM-kinase II with Ser^{282} and Ser^{272} as possible targets (Fleischman et al., 1993). Our data show that Ser^{282} is indeed a major calcium-responsive phosphorylation site of Ets1 in vivo but rule out a role of Ser^{272}, since mutation of this residue into alanine in Ets1-mut6 does not change the tryptic phosphopeptide pattern of Ets1. Besides CaM-kinase II, which is involved in the response of CaM-kinase response element to CAAT/enhancer-binding protein β (Wegner et al., 1992), other members of the CaM-kinase family, such as CaM-kinase IV (Ohmstedt et al., 1989), have been implicated in calcium-dependent transcriptional regulation of cAMP-response element-dependent transcription (Enslen and Soderling, 1994). Since CaM-kinase IV has a significant nuclear localization (Jensen et al., 1991), and since its activity has been shown to increase following TCR engagement in Jurkat T cells (Hanjian et al., 1993), CaM-kinase IV might be involved in Ets1 phosphorylation at Ser^{282}. Experiments are in progress to identify which member(s) of this family of kinases is actually responsible for Ets1 phosphorylation. Although essential, phosphorylation of Ser^{282} is clearly insufficient to the calcium-dependent inhibition of DNA binding which also requires the simultaneous phosphorylation of Ser^{285}, Ser^{281}, and Ser^{287}. These serine residues are found in a similar primary sequence environment (D/E)[S/F/Y](D/E)] which may correspond to a consensus recognition sequence for a novel type of protein kinase. This protein kinase does not necessarily depend upon calcium for its activity since our experiments did not address the question of whether the calcium-dependent phosphorylation of Ets1 occurs by up-regulation of protein kinase(s) or by down-regulation of protein phosphatase(s).

The c-ets1 exon 7-encoded domain has been implicated in the intramolecular repression of Ets1-specific DNA binding activity in vitro (Lim et al., 1992; Waslyly et al., 1992). None of the serine to alanine mutations analyzed here are sufficient to activate Ets1 DNA binding activity, suggesting that basal phosphorylation of the exon 7-encoded serine residues is not involved in intramolecular inhibition of DNA binding. The calcium-induced phosphorylation of Ets1 as it occurs during T and B cell activation appears therefore to superimpose an additional level of control on the activity of the inhibitory domain. These phosphorylation events could for example shift the equilibrium among Ets1 conformers from a DNA binding competent to an incompetent state. Such locking of Ets1 in a conformation incompetent for DNA binding could result from specific interactions between the phosphate groups of target serine residues, e.g. with basic amino acids in the Ets domain which are essential to DNA binding activity (Wang et al., 1992; Bosselut et al., 1993).

The activity of several Ets family members has been shown to be modulated by phosphorylation events. Phosphorylation of Ser^{148} of Pu-1 by casein kinase II, although not affecting Pu-1 DNA binding activity, is essential to the ability of Pu-1 to re-

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FIG. 9. Subcellular localization studies. Panel A, HeLa cells transfected with either the control expression plasmid or the expression plasmid encoding tagged Ets1 were seeded on collagen-treated coverslips and processed for immunofluorescence analysis. Prior to fixation, cells were either left untreated or were treated for the indicated times with 2 μM ionomycin. The top of the figure shows an immunoblotting analysis of the same cells using the 12CA5 monoclonal antibody. Panel B, Jurkat cells transfected with the expression plasmid encoding the tagged Ets1 were either left untreated or were treated for 5 min with 2 μM ionomycin before processing for immunofluorescence analysis using the 12CA5 monoclonal antibody.

Control | Ets-1
---|---
No treatment | 5 min. Iono. | 30 min. Iono. | 45 min. Iono.
Phase contrast | 5 min. Iono.
Fluorescence
cruit the B cell restricted factor NF-EM5 to a composite DNA element in the immunoglobulin κ3 enhancer (Pongubala et al., 1993). The packaging of the C box of Elk-1 by mitogen-activated protein kinase has been shown to be required for transcriptional activation by Elk-1, but not to its intrinsic DNA binding activity or its ability to interact with SRF on the c-fos SRE (Marais et al., 1993; Hill et al., 1993; Janknecht et al., 1993).

Elts is also a substrate for mitogen-activated protein kinase, since it is efficiently phosphorylated by mitogen-activated protein kinase in vitro on threonine residue 38, which also becomes phosphorylated in vivo in response to serum activation in fibroblasts. A similar activity is likely to be responsible, at least in part, for the induced phosphorylation observed here in Jurkat T cells for mutants like Elts1-mut9 or Elts1 2443-330 in response to TCR engagement. In contrast to these situations, the calcium-dependent phosphorylation events observed in response to TCR engagement affect the ability of Elts1 to bind its specific DNA response elements. Elts is expressed at high levels in quiescent circulating T cells as well as nonactivated T cell clones and cell lines (Pongueone et al., 1988, 1990; Koizumi et al., 1990; Bhat et al., 1991) in a form containing both Elts and Elts1 binding sites are present in T cell-specific promoters and/or enhancers, including the TCR α and β chain enhancers (Ho et al., 1990; Prosser et al., 1992), the CD4 promoter (Salmon et al., 1993), the IL-2 Rβ chain promoter (Lin et al., 1993), and the lck type I and type II promoters (Leung et al., 1993; Gégonne et al., 1993). Our data show that following binding of antigen to its receptor or following other ligand-receptor interactions which also result in [Ca2+]i increases, Elts1 is expected to exist in a DNA incompetent form. In addition, expression of c-ets1 has been shown to be down-regulated to undetectable levels within 2 h of T cell activation (Bhat et al., 1990). The calcium-mediated phosphorylation events analyzed here represent therefore the first level of control of a general clearance mechanism for Elts1 function in the early stages of T cell activation. Elts1 could function as a tissue-specific activator of genes such as those encoding CD4, IL-2 Rβ, or Lck known to be expressed in nonactivated T cells or as a repressor of genes normally up-regulated in the early stages of activation. Clearance of Elts1 function as it occurs upon T cell activation could lead either to simple relief of repression and/or, in part, for the induced phosphorylation observed here in Jurkat cell elec-

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