ERM Transactivation Is Up-regulated by the Repression of DNA Binding after the PKA Phosphorylation of a Consensus Site at the Edge of the ETS Domain*

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The final step of the transduction pathway is the activation of gene transcription, which is driven by kinase cascades leading to changes in the activity of many transcription factors. Among these latter, PEA3/E1AF, ER81/ETV1, and ERM, members of the well conserved PEA3 group from the Ets family are involved in these processes. We show here that protein kinase A (PKA) increases the transcriptional activity of human ERM and human ETV1, through a Ser residue situated at the edge of the ETS DNA-binding domain. PKA phosphorylation does not directly affect the ERM transcriptional domains but does affect DNA binding activity. Unphosphorylated wild-type ERM bound DNA avidly, whereas after PKA phosphorylation it did so very weakly. Interestingly, S367A mutation significantly reduced the ERMediated transcription in the presence of the kinase, and the DNA binding of this mutant, although similar to that of unphosphorylated wild-type protein, was insensitive to PKA treatment. Mutations, which may mimic a phosphorylated serine, converted ERM from an efficient DNA-binding protein to a poor DNA binding one, with inefficiency of PKA phosphorylation. The present data clearly demonstrate a close correlation between the capacity of PKA to increase the transactivation of ERM and the drastic down-regulation of the binding of the ETS domain to the targeted DNA. What we thus demonstrate here is a relatively rare transcription activation mechanism through a decrease in DNA binding, probably by the shift of a non-active form of an Ets protein to a PKA-phosphorylated active one, which should be in a conformation permitting a transactivation domain to be active.

The regulation of gene expression by specific signal transduction pathways is tightly connected to cell phenotype. Several molecules involved in intracellular signaling are encoded by oncogenes, which directly link their potential aberrant expression to cell transformation or altered proliferation. The final step of the transduction pathway is the activation of nuclear transcription factors. For example, the cyclic-AMP- and calcium-regulated nuclear factor is activated through the protein kinase A (PKA) pathway via phosphorylation. The differential phosphorylation of transcription factors by signal transduction pathways such as the mitogen-activated protein kinase (MAPK) plays a crucial role in the regulation of gene expression. This is the case for the c-fos gene expression, which is regulated by the binding to DNA of the Elk/TCF factor after phosphorylation through the MAPK pathway. The activation of MAPK cascades leads to changes in the activity of many Ets factors such as Elk/TCF (for reviews, see Refs. 1 and 2).

The Ets family of transcription factors, which includes more than 30 members from sponges to humans (for reviews, see Refs. 2 and 3), has been involved in both tumorigenesis and a number of developmental processes. They all contain the ETS domain (4), a domain of 85 amino acids structured as a winged helix-turn-helix structure and responsible for DNA binding to the specific core sequence GGA(A/T) (for review, see Refs. 2). These factors can be subclassified primarily because of the amino acid conservation in their ETS domains and also because of the conservation of other domains generally characterized as transactivating.

In the case of the PEA3 group, which is made up of three members, PEA3 (also called E1AF in the human or ETV4) (5, 6), ER81 (also called ETV1 in the human) (7–9), and ERM (also called ETV5) (10–12), these factors are more than 95% identical in the ETS domain and more than 85% in the NH2-terminal 32 residue acidic domain, and almost 50% identical in the final 61 residues corresponding to the carboxyl-terminal tail of the proteins (carboxyl-terminal domain) (for review, see Ref. 13). The transactivating activity of these factors is because of the two conserved acidic and carboxy-terminal domains (11, 14–16).

Although the putative target genes of these three transcription factors are multiple, their most frequently studied role concerns their involvement in breast cancer metastasis (17, 18), probably by activating the transcription of matrix metalloproteinases (19, 20). Concerning their expression in the adult, although pea3/e1af and er81/etv1 display more restricted expression patterns (5, 7–10, 12), erm has been characterized as a more ubiquitously expressed gene with its highest expression in the brain (10). Recently, it has been demonstrated that ERM is induced by interleukin-12 through a Stat4-dependent pathway in Th1 and could play a highly specific role by inducing Th1 differentiation in the mouse (21).

The transcription capacities of mouse and zebrafish PEA3, ¹ The abbreviations used are: PKA, protein kinase A; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay.
mouse and human ER81/ETV1, and human ERM are increased by components of the MAPK cascades, Ras, Raf-1, MEK, and the MAPK ERK-1 and ERK-2; this suggests that these factors may contribute to the nuclear response to cell stimulation and Ras-induced cell transformation (15, 16, 22, 23). Interestingly, PKA is also able to increase the transcriptional activity of human ERM (15), human ETV1 (24), and zebrafish PEA3 (23) probably through phosphorylation since we have previously demonstrated that ERM can be phosphorylated in vitro by active PKA (15). In this study we show that, in contrast to human ERM and ETV1, PKA is not able to significantly increase the transcriptional activity of mouse PEA3. In fact, this transcription factor lacks a classical PKA phosphorylation site present at the beginning of the ETS domain of ERM and ETV1; this domain is phosphorylated by PKA, and is necessary for the activation of ERM by the kinase. We further show that the increase in ERM transcriptional activity after PKA phosphorylation is closely correlated with a drastic reduction in the DNA binding of the transcription factor. These results indicate that the phosphorylation of ERM by PKA is involved in ERM-mediated transcription and suggest that the activation of ERM is probably related to conformational changes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Rabbit kidney epithelial-like RK13 cells (ATCC number CCL 37), HeLa and COS-7 cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in Dulbecco’s medium supplemented with 10% fetal calf serum (Invitrogen)

**Plasmids**—The 3xET74-kt-Luc reporter plasmid and the overexpression vectors for the catalytic subunit of PKA have been described previously (15, 25). The plasmid −517/+63 Coll-Luc containing the indicated region of the human collagenase 1 promoter sequence linked to a luciferase gene (20) has been described.

The full-length region of human ERM (10), human ETV1 (8), and mouse PEA3 (5) were amplified by PCR, digested, and subcloned in the EcoRI and BglII sites of the pSV vector, an expression vector which provides an amino-terminal hemagglutinin tag (14), to produce pSV-ERM, pSV-ETV1, and pSV-PEA3, respectively. The point mutations of ERM, pSV-ETV1, and pSV-PEA3, respectively. The point mutations of ERM provides an amino-terminal hemagglutinin tag (14), to produce pSV-RI and pSV-II sites of the pSV-ERM and pSV-PEA3, respectively. The point mutations of ERM were generated by site-directed mutagenesis (23)

**RESULTS**

**ERM Transactivation Is Up-regulated by PKA Phosphorylation**

The full-length region of human ERM (10), human ETV1 (8), and mouse PEA3 (5) were amplified by PCR, digested, and subcloned in the EcoRI and BglII sites of the pSV vector, an expression vector which provides an amino-terminal hemagglutinin tag (14), to produce pSV-ERM, pSV-ETV1, and pSV-PEA3, respectively. The point mutations of ERM, pSV-ETV1, and pSV-PEA3, respectively. The point mutations of ERM provides an amino-terminal hemagglutinin tag (14), to produce pSV-RI and pSV-II sites of the pSV-ERM and pSV-PEA3, respectively. The point mutations of ERM were generated by site-directed mutagenesis (23)

**Activation of the PEA3 Group Members by PKA**—Although human ERM and ETV1 are targeted by the PKA signaling pathway (15, 24), no information is available concerning the transactivation of mammalian PEA3 through PKA activation.
We therefore began by comparing the effect of the PKA pathway on the transactivation capacities of human ERM, human ETV1, and mouse PEA3 in transient transfection experiments. Based on previous experiments showing that the E74 reporter plasmid is not activated by PKA in RK13 cells (15, 24), we used this system to compare the activation of the PEA3 group members by the kinase. In the absence of a PKA expression vector none of the three proteins was found to stimulate the transactivation of the E74 reporter plasmid (Fig. 1). In contrast, the expression of these transcription factors led to a 30–70% transcription repression when compared with the basal level. The co-transfection of a PKA expression vector did not increase the basal transactivation on the E74 reporter. However, as previously reported, the expression of PKA significantly increased transactivation when ETV1 or ERM were coexpressed. Concerning PEA3, the coexpression of PKA only abolished the repression on the reporter since no significant increase in transactivation was observed as compared with the basal level. These data clearly show that of the three mammalian PEA3 group members, only ERM and ETV1 are targeted by the PKA pathway on the E74 reporter.

Mapping of the Major ERM Phosphorylation Sites—As previously described (15), ERM is phosphorylated in vitro by purified PKA. The examination of the ERM amino acid sequence for phosphorylation consensus sites by known kinases revealed a single consensus PKA phosphorylation site (RRGS\textsuperscript{367}) in a region located at the beginning of the DNA-binding ETS domain. Furthermore, the amino acid sequence of the region including this site is well conserved among the PEA3 group members of different species. Interestingly, only mouse and human PEA3 contain an alanine substitution at the putative serine phosphoacceptor site (RRGA, Fig. 2\textsuperscript{A}). Since PKA is also able to putatively phosphorylate other sites present in the protein (RXXS or RXS (28)), we first determined the regions of ERM that are phosphorylated in vitro by this kinase. We thus expressed human ERM (aa 12–510) and mutants lacking portions of the NH\textsubscript{2} or COOH termini as GST fusion proteins in Escherichia coli (Fig. 3\textsuperscript{A}). Partially purified proteins were then tested for their ability to serve as substrates for purified PKA (Fig. 3\textsuperscript{B}).

The results indicate that phosphorylation occurred at the COOH-terminal end of ERM since ERM-(354–510), which contains the consensus PKA phosphorylation site, was an excellent substrate for the kinase (Fig. 3\textsuperscript{C}). However, the proteins of the 363 and 298 residues obtained by the COOH-terminal truncation of ERM were also efficiently phosphorylated by the kinase, whereas further COOH-terminal truncation of ERM to a protein with 226 residues led to a severe reduction in phosphorylation. Accordingly, when ERM-(199–283) was used as a substrate, there was efficient phosphorylation of the fusion protein, and only the GST moiety was not phosphorylated (data not shown). Thus, by deletion analysis, the PKA phosphorylation domains of ERM were located both at the COOH-terminal region including the consensus PKA phosphorylation site (Ser\textsuperscript{367}), and at a central region between residues 226 and 298. This latter domain contains two contiguous potential sites for PKA phosphorylation (Arg-Pro-Ser\textsuperscript{242}-Tyr-His-Arg-Gln-Met-
Ser^{245}), the first being specific to human and mouse ERM and the second shared by ERM and ETV1 (Fig. 2B).

To specify the PKA phosphorylation sites, mutations within the mapped regions were generated in the GST-ERM protein, i.e. GST-ERM S367A, in which the serine indicated was mutated into an alanine, GST-ERM Δ, in which the 226–250 region was deleted and GST-ERM Δ S367A, which combined the two mutations. When compared with GST-ERM, a significant decrease in ^32P incorporation was observed in the GST-ERM S367A following incubation with PKA (Fig. 4A, compare lanes 1 and 2). Similarly, the GST-ERM Δ exhibited substantially less total phosphorylation (lane 3). A combination of the two mutations eliminated nearly all the ERM phosphorylation (lane 4). Since a Coomassie Blue stain of the same gel indicated that approximately equal amounts of the four substrates were present in the kinase reaction (Fig. 4B), these data show that the in vitro PKA phosphorylation of ERM is mainly dependent on Ser^{245} and Ser^{248}. In vitro PKA assay with wild-type and ERM mutants as substrates. A, ERM-(12–510) (ERM wt), ERM S367A, ERM Δ226–250 (ERM Δ), and ERM Δ226–250 S367A (ERM Δ S367A) produced as GST fusion proteins were phosphorylated in vitro by PKA and analyzed by SDS-PAGE and autoradiography. The molecular masses are shown on the left. B, Coomassie Blue staining of the same gel.

**Mutation of Ser^{367} Phosphorylation Site Reduces ERM Transcriptional Capacity by PKA**—To analyze the functional role of phosphoacceptor sites in the activity of ERM protein we assessed whether the mutated proteins were still transcriptionally active in the presence of PKA. In the absence of the PKA expression vector, the co-transfection of RK13 cells with the E74 reporter and plasmids expressing either wild-type ERM or mutant ERMs (ERM S367A, ERM Δ, and ERM Δ S367A) resulted in similar levels of luciferase activity, which were slightly lower than the basal level (Fig. 5A). In contrast, when the catalytic subunit of PKA was coexpressed with GST-ERM, wild-type ERM gave a 7.4-fold transcription activation in relation to ERM alone, which was very similar to that seen with ERM Δ (7.7-fold activation). However, a significantly lower level of transcription activation was observed when Ser^{367} was mutated into alanine, i.e. in cells expressing ERM S367A (3.2-fold activation) and ERM Δ S367A (2.3-fold activation). Under the conditions used for the transcriptional assays it was very difficult to determine the expression level of the wild-type and mutant ERM proteins. Nevertheless, an immunoprecipitation analysis of ^35S-radiolabeled RK13 cells co-transfected with higher concentrations of the ERM effectors demonstrated that the Ser^{367} mutation did not affect the relative expression level of the mutated ERM protein compared with the expression level of the wild-type protein in either the absence or presence of coexpressed PKA (Fig. 5B). Taken together, these data indicate that whereas mutating Ser^{367} to alanine results in a protein with a reduced transcriptional enhancement capacity by PKA, the PKA phosphorylation site located in the central part of the protein does not affect ERM in response to the kinase. Furthermore, immunoprecipitation assays were also performed on co-transfected cells labeled with ^32Porthophosphate to investi-
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Fig. 6. Comparison of the effect of PKA on the transactivation of E74 and collagenase 1 promoters by wild-type and mutant ERM in RK13 and HeLa cells. Expression plasmids for ERM wt or ERM S367A (ERM S/A) were co-transfected in RK13 cells with the E74 or the −157/+63 collagenase 1 (Coll1) reporter plasmids in both the absence (−) or presence of the PKA expression plasmid as described in the legend to Fig. 5. Transient co-transfections of HeLa cells were performed as described above with the pEFIN3-ERM or pEFIN3-ERM S367A expression vectors.

PKA Does Not Directly Affect the ERM Transactivation Domains—ERM was shown to contain two transactivation domains: the NH2-terminal part of the protein including the acidic domain (amino acids 1–72), and the last 61 residues of the protein (14). We had previously reported that the deletion of the COOH-terminal transactivation domain did not affect the PKA-dependent activation of ERM. In contrast, the deletion of the NH2-terminal transactivation domain nearly eliminated the transcriptional activation of the protein in the presence of PKA (15). We therefore investigated whether the ERM NH2-terminal transactivation domain was capable of responding to PKA when fused to a heterologous DNA-binding domain, i.e. that of the yeast protein GAL4. We consequently fused full-size ERM and various C-terminally truncated mutants to the GAL4 DNA-binding domain and tested the transcriptional activity in the context of a luciferase reporter construct driven by GAL4-binding sites. A marked increase in basal transcription in the RK13 cells was observed with the GAL4-(1–72) and GAL4-(1–122) constructs. However, the expression of PKA exhibited no significant effect on transactivation (Fig. 7A). This agrees with the fact that no PKA phosphorylation site has been identified in the NH2-terminal region. As reported before, a dramatic reduction of activation was observed from GAL4-(1–81) to GAL4-(1–370), a phenomenon probably because of the presence of a negative regulatory domain in the region from residues 166 to 326 (11). Again, the coexpression of PKA did not affect the activation potential of these fusion proteins even in the case of GAL4-(1–370), which contains the Ser367 phosphorylation site. Finally, GAL4-(1–510), which is transcriptionally inactive, was also unresponsive to PKA (Fig. 7A). However, when this fusion protein of the GAL4 DNA-binding domain and full-length ERM was assayed with the Coll1 reporter, it was active and a PKA-mediated activation was observed. Furthermore, mutation of Ser367 into Ala in the fusion protein resulted in a large decrease in transcription activity only when PKA was expressed in the transfected cells (Fig. 7B). It thus seems likely that the ERM NH2-terminal transactivation domain is not directly targeted by PKA, and that the kinase is able to activate ERM in the context of GAL4 fusion proteins when tested with a GAL4-binding site-driven reporter.

PKA Phosphorylation Affects the DNA Binding Activity of ERM—Because of the fact that the functional PKA phosphorylation site of ERM is situated at the edge of the ETS domain, we wondered whether PKA phosphorylation alters the capacity of ERM to interact with DNA. For this study we used gel mobility shift assays with the full-length GST-ERM protein, which was first subjected to different kinase reactions and then used for DNA-binding assays. As in the absence of PKA (Fig. 8A, lanes 1–2), in the presence of PKA but in the absence of ATP (lane 3), ERM was able to interact with an E74 oligonucleotide probe. However, the binding of ERM was nearly eliminated (lane 4) upon the addition of ATP and PKA, and addition of the PKA inhibitor PKI to the phosphorylation reaction eliminated
the effect of PKA on the DNA binding activity of the wild-type ERM (lane 5). It must be noted that a remaining DNA binding was observed for the PKA-phosphorylated ERM when a higher amount of protein was used (Fig. 8B). In the two remaining ERM-DNA complexes detected, the faster migrating complex most probably corresponded to the remaining unphosphorylated ERM. On the other hand, the slowly migrating complex was specifically observed after the PKA phosphorylation, so indicating that phosphorylation may have induced a conformational change in ERM.

These results thus strongly suggest that phosphorylation causes the large decrease in DNA binding activity. However, it was not possible to deduce from our in vitro analysis whether phosphorylation at Ser367 was solely responsible for the change in DNA binding activity since in vitro, PKA phosphorylates another site in the central part of ERM. For this reason we examined whether phosphorylation by PKA was able to alter the capacity of ERM S367A to interact with DNA. As shown in Fig. 8A the incubation of GST-ERM S367A with PKA and ATP nearly eliminated the DNA binding activity of wild-type ERM (Fig. 8D, compare lanes 1 and 3), while the binding of ERM S367A was apparently unchanged (compare lanes 2 and 4). It must, however, be noted that we always observed a lower degree of DNA binding in the case of wild-type ERM when compared with mutated ERM. This difference in DNA binding of the reticulocyte proteins was not because of less wild-type ERM because the amounts of protein used were equalized through normalization to [35S]methionine incorporation (data not shown). Since this difference was not detected with bacterially produced proteins, this finding suggests that DNA binding might be modulated by Ser367 phosphorylation in reticulocytes. To test this hypothesis the two reticulocyte proteins were treated with alkaline phosphatase prior to gel shift assays. As illustrated in Fig. 8E, whereas dephosphorylation treatment did not significantly alter binding by the serine to alanine mutant (compare lanes 2 and 4), it enhanced the DNA binding capacity of wild-type ERM when compared with mutated ERM. This difference in DNA binding of the reticulocyte proteins was not because of less wild-type ERM because the amounts of protein used were equalized through normalization to [35S]methionine incorporation (data not shown). Since this difference was not detected with bacterially produced proteins, this finding suggests that DNA binding might be modulated by Ser367 phosphorylation in reticulocytes.

EMSAs were finally performed to determine whether PKA regulated binding of ERM in transfected cells. Comparison was made using equal amounts of nuclear extracts derived from...
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FIG. 8. The effect of the in vitro phosphorylation by PKA of ERM on DNA binding activity. A, bacterially produced GST-ERM-(12–510) and GST-ERM-(12–510) S367A were treated in the presence (+) or absence (−) of purified PKA with or without ATP or the PKA inhibitor PKI, and tested in gel mobility shift assays using an E74 double strand oligonucleotide probe. B, GST-ERM-(12–510) was treated and analyzed as in A except that the experiment was performed with double the amount of proteins. C, bacterially produced GST-ERM-(12–510) and GST-ERM-(12–510) S367A were treated in the presence (+) or absence (−) of purified PKA and tested in gel mobility shift assays using a collagenase 1 double strand oligonucleotide probe. D and E, full-size ERM and its S367A mutant were produced in reticulocyte lysate, incubated in the presence (+) or absence (−) of PKA (D) or alkaline phosphatase (E) and tested in gel mobility shift assays using an E74 probe.

COS-7 cells transfected with expression vectors encoding either wild-type ERM or ERM S367A alone or together with a PKA expression plasmid (Fig. 9). In comparison to control cells (lane 1, Fig. 9A), a band was detected in nuclear extracts of cells transfected with wild-type ERM or ERM S367A alone (lanes 2 and 3) and these complexes bound to the E74 probe were supershifted by the addition of an ERM antibody (Fig. 9B). However, when PKA was co-expressed in transfected cells, the binding of wild-type ERM was significantly reduced (lane 5, Fig. 9, A and B) whereas that of ERM S367A was unaffected (lane 6, Fig. 9, A and B). This result was not because of less wild-type ERM in cells co-transfected with PKA since immuno blot analysis revealed equivalent levels of protein expression for wild-type ERM and ERM S367A in PKA-transfected cells (Fig. 9C). Taken together, all the results are consistent with the notion that PKA-mediated phosphorylation of Ser367 acts to negatively regulate the DNA binding activity of ERM.

DNA Binding Capacity of the PEA3 Group Members Phosphorylated by PKA—The above results indicate that Ser367 phosphorylation by PKA changes the DNA binding capacity of ERM. Since the site for PKA phosphorylation identified in ERM was present at the same location in human ETV1 but absent in human PEA3 (Fig. 2A), we evaluated the possible effects of PKA phosphorylation on the DNA binding of these other two PEA3 group members. To this end full-length ERM, ETV1, and PEA3 were synthesized in rabbit reticulocyte lysate, treated with or without purified PKA and subjected to gel shift assays. ETV1 and PEA3 bound to the E74 probe after treatment with or without PKA (Fig. 10A, left-hand panel) as did ERM. It must, however, be observed that PEA3 bound to DNA more avidly than ERM or ETV1. This difference was not because of more PEA3, since the experiment was performed with roughly equal amounts of the proteins (Fig. 10B). Furthermore, a significant difference in DNA binding was still observed after alkaline phosphatase treatment of the three reticulocyte-translated proteins, so indicating that the lower binding capacity of ERM and ETV1 as compared with PEA3 cannot be explained by the phosphorylation of these two proteins in reticulocytes (data not shown). After in vitro PKA treatment, similar results were obtained for ERM and ETV1, i.e. phosphorylation by the kinase eliminated the DNA binding. On the other hand, the DNA binding of PEA3 following PKA treatment was similar to that of the untreated protein (Fig. 10A, right-hand panel). It thus appears that ERM and ETV1, both of which are transcriptionally activated by PKA, show altered properties in DNA binding following in vitro PKA phosphorylation whereas PEA3, which is not significantly transcriptionally activated by PKA, is not affected in its DNA binding after PKA treatment.

Effect of Mutation of Ser367 into Acidic Residues on ERM Properties—Since the phosphorylation of Ser367 influences the capacity of ERM DNA binding activity, we employed site-directed mutagenesis to change this phosphoacceptor site into either aspartic acid or glutamic acid, which may mimic a phosphorylated serine. The mutated cDNAs encoding full-length ERM S367D and ERM S367E were transcribed and translated in vitro, and the mutant proteins were examined for DNA binding. In contrast to the result obtained for ERM S367A (Fig. 11A, lane 2), we found that the substitution of serine for glutamic acid converted ERM from an efficient DNA-binding protein into a poor DNA binding one (compare lanes 1 and 3). Similar data were obtained with a mutated protein created by
inserting aspartic acid in place of serine (data not shown), and the variation observed in binding between wild-type and mutant proteins was not because of differences in protein amounts (Fig. 11B). When the proteins were submitted to PKA phosphorylation before the gel shift assays, no significant change in DNA binding was observed for the two mutated proteins ERM S367A and ERM S367E, while the binding of wild-type ERM was nearly eliminated (Fig. 11A). This confirms that mutating Ser<sup>367</sup> into non-phosphorylatable residues results in mutated proteins unresponsive to PKA in terms of DNA-binding capacity and clearly shows that inserting acidic residues in place of Ser<sup>367</sup> decreases ERM DNA binding, but not to the same extent as after the PKA-dependent phosphorylation of the wild-type protein.

We also tested whether the substitution of serine for glutamic acid in ERM led to changes in the transcriptional activation of the E74 reporter in RK13 and HeLa cells. In the absence of the PKA expression vector, the expression of ERM S367E led to a weak activation of transcription as compared with wild-type ERM (Fig. 11C). Further activation was observed in cells co-transfected with the PKA expression vector. However, the degree of activation in the presence of PKA was lower than that obtained for wild-type ERM, but greater than that observed for ERM S367A (compare Fig. 11C and Fig. 6). Similar results were also obtained with ERM S367D (data not shown). These results indicate that: 1) the substitution of serine for glutamic or aspartic acid leads to an increase in transcriptional activity for the mutated proteins in the absence of PKA, but is not sufficient to mimic serine phosphorylation; and 2) the variations in ERM-mediated E74 activation by PKA are closely correlated with changes in DNA binding activity. However, as illustrated in Fig. 11C, mutation of Tyr<sup>419</sup> to Pro in the ETS domain of ERM, which abolishes DNA binding (14), generated a protein unable to activate transcription in the presence or absence of PKA. This indicates that ERM binding is a prerequisite for transcriptional activation by PKA.

**DISCUSSION**

In the present investigation, we confirm that ERM is targeted by the PKA pathway, and we show that of the two other known mammalian members of the PEA3 group of Ets-related transcription factors, i.e. ETV1 and PEA3, only ETV1 is significantly activated transcriptionally by PKA. Our results indicate that Ser<sup>367</sup> plays a crucial role in the transcriptional activation of ERM by PKA inasmuch as its mutation into non-phosphorylatable alanine significantly reduces ERM-mediated transcription in the presence of the kinase. This residue, which is in a favorable consensus sequence, is phosphorylated by PKA in *in vitro* as well as *in vivo* and, interestingly, the corresponding region in mouse ETV1 is also highly phosphorylated by PKA in *in vitro* (16). In fact, this region of the protein is well conserved in the three PEA3 group members. However, while the RRGSS<sup>367</sup> motif of this region is conserved in ERM and ER81/ETV1, a substitution of serine for alanine is found in mouse and human PEA3, thus strongly suggesting that the presence of this site is necessary for the transcriptional activation of PEA3 group members by PKA. In support of this conclusion there is the recent observation that zebrafish PEA3, which contains this consensus site along with the serine residue, is activated transcriptionally in the presence of PKA (23).

We show here that the phosphorylation of ERM by PKA...
greatly decreases the amount of protein bound to Ets-binding sites. This effect is due specifically to the phosphorylation of Ser\(^{367}\) since no change in DNA binding activity is observed after the phosphorylation of ERM S367A. Since the phosphorylation site is located near the DNA-binding domain, we envisaged that phosphorylation could interfere with DNA binding by electrostatic repulsion, or by inhibiting contacts between the protein and the DNA by steric hindrance. To examine these possibilities, we replaced Ser\(^{367}\) with either aspartic or glutamic acid in an attempt to mimic phosphorylation in the ERM molecule. Both mutant proteins had the same DNA-binding capacity and were insensitive to PKA action, so confirming the role of Ser\(^{367}\) in the change of ERM DNA binding activity upon PKA phosphorylation. However, both ERM mutants exhibited lower DNA binding activity as compared with the wild-type non-phosphorylated protein, but the effect was not as pronounced as that of the phosphorylation of Ser\(^{367}\) in the wild-type protein. This indicates that not only phosphorylation can act by electrostatic repulsion and/or steric hindrance, but also that other mechanisms may be key determinants. Particularly, structural differences between phosphorylated and non-phosphorylated ERM may be critical for DNA binding. Indeed, we have shown that the phosphorylation of ERM by purified PKA alters the mobility of the remaining ERM-DNA complexes, so suggesting that the DNA-binding conformations before and after phosphorylation are distinct. Furthermore, conformational rearrangements have been shown to be related to the regulation of DNA binding in Ets proteins. A more extensively studied example is Ets-1, whose DNA binding activity is inhibited by a module consisting of two a-helices located NH\(_2\)-terminal to the ETS domain, and a single COOH-terminal a-helix. A conformational change involving the unfolding of one of the NH\(_2\)-terminal helices is associated with the relief of this intramolecular inhibition (29, 30). This conformation is thought to be stabilized through protein interactions with inhibitory regions (29, 31). Furthermore, it has been shown that the phosphorylation of Ets-1 in the NH\(_2\)-terminal inhibitory region results in the inhibition of Ets-1 binding to DNA (32–34), favoring a shift of the conformational equilibrium toward a folded state (35). It has also been proposed that the phosphorylation of Elk-1 induces a conformational change in its structure, which affects its ability to bind DNA (36–38).

While the ability of PKA-phosphorylated ERM to bind DNA in vitro is decreased, PKA stimulates ERM-dependent transcription in transient transfected cells. In fact, the ability of ERM to bind DNA is correlated with its transactivation activity. First, ERM and ETV1 avidly bound the E74 probe, although the proteins did not or modestly transactivate the E74 reporter, while an important decrease in DNA binding for the PKA-phosphorylated proteins is associated with PKA-mediated transactivation in transfected cells. In contrast, PEA3, which also avidly binds the probe but is unresponsive to PKA in terms of DNA binding, is not significantly transcriptionally activated in the presence of PKA. Second, the mutation of Ser\(^{367}\) into alanine eliminates the apparent reduction in DNA binding following PKA phosphorylation and leads to a marked decrease of ERM transcriptional activation in the presence of PKA. Finally, the mutation of Ser\(^{367}\) into aspartic acid or glutamic acid leads to the formation of an ERM protein with a moderate reduction in DNA binding and which is transcriptionally more active than the wild-type protein. Interestingly, this latter observation has been made on transfected cells in the absence of PKA, thus indicating that the decrease in DNA binding and the increase in transcriptional activity are intimately related. However, the interaction of ERM with DNA is essential since a mutant version of ERM which does not bind DNA because of a single mutation in its ETS-domain (ERM Y419P (14)) is transcriptionally inert in the absence or presence of PKA.

Although often described (25, 39), increased transcriptional activation does not necessarily correlate with enhanced transcription factor binding to a promoter. In a recent work (40) on the transcription factor B-Myb, it has been shown that mutation of a single phosphorylation site enhanced binding to a Myb-binding sequence but decreased B-Myb transactivation potential. Furthermore, there is growing evidence that high affinity binding does not always lead to transactivation. Thus, some NF-\(\kappa\)B DNA-binding sites have been found to be very poorly activated by NF-\(\kappa\)B proteins (41) and high affinity binding of GATA-1 to a reporter gene does not necessarily induce transactivation (42). Concerning Ets proteins, it has recently been described that some natural promoter sites do not correspond to high consensus sites derived from in vitro studies of isolated Ets proteins (43). Moreover it has been observed that the Ets protein Erg activates the collagenase 1 promoter while any binding of the Erg protein is detected to the promoter, whereas the protein binds to the stromelysin I promoter but does not activate this Ets-regulated gene (44). A major factor that plays a role in Ets protein-DNA recognition is cooperative protein-protein interactions between Ets proteins and other factors that can alter the affinity of the Ets proteins for DNA (2). Thus, considering the ability of transcriptional regulators to adapt their structures to the particular DNA sequence that they recognize (42, 45, 46), it is possible that an Ets protein can bind to some Ets-binding sites without engaging protein-protein contacts or appropriate interactions with protein partners. This would induce an inability or a poor capacity to activate transcription. We therefore propose that phosphorylation of the transcription factor may affect its activity by altering its ability to interact with DNA and other proteins. In this model, the events which lead to decreased ERM DNA-binding capacity would result in conformational changes of the ERM molecule favoring its association with proteins involved in transcription. As suggested above, it is likely that decreased DNA binding of phosphorylated ERM is at least partially because of structural change. Furthermore, it cannot be excluded that a change in the DNA binding activity of phosphorylated ERM might lead to an altered conformation of the ERM-DNA complex affecting the protein interaction capacities of the bound protein. This would explain why the GAL4/ERM fusion protein is unresponsive to PKA when assayed with the GAL4-binding site-driven reporter while it is activated by the kinase when tested on an Ets-binding site reporter. Thus, enhanced protein-protein interactions could stabilize the interaction of the protein with DNA to overcome the decrease in DNA binding of the PKA-phosphorylated protein and favor the formation of a multiprotein complex to activate transcription.

A comparison in transfected cells of the properties of wild-type ERM with those of mutant versions carrying Ser\(^{367}\) for alanine substitutions has shown that this site is necessary for mediating a full transcriptional response with respect to PKA. However, mutating this site is insufficient to fully eliminate the effect observed since residual activation is still detected in the mutated proteins as compared with the wild-type protein in the absence of PKA. This residual activation cannot be attributed to the phosphorylation of the other PKA site identified in the central part of the protein since mutant ERM carrying an Ser\(^{367}\) for alanine substitution and the deletion of the second PKA-targeted region is still weakly activated transcriptionally when the kinase is expressed. An explanation would be that the residual transactivation effect is mediated by a PKA-activated kinase. Indeed, PKA may activate the MAP kinase ERK via a Ras-independent path-
way (47), and ERK regulates several downstream signaling events, including the function of the ER transcription factor (15). However, the inhibitor PD 98059, previously shown to selectively inhibit the ERK1/ERK2 MAPK pathway (48), did not reduce the PKA-induced ERK transcriptional activity (data not shown). Another possibility is that PKA may activate protein partners acting in concert with the PEA3 group members to activate transcription. This would explain why PEA3 which does not contain the PKA site is weakly activated by PKA. It must also be mentioned that the PKA-mediated activation of the ER binding site occurs similarly in both RK13 and HeLa cells but that this effect is more pronounced on the Coll1 promoter as compared with the E74 one (about 50 and 25% of wild-type ER activation, respectively). An activation mediated indirectly through other proteins might thus be promoter-dependent and the activation level might reflect differences in the ability of ERM to interact with these proteins depending of the binding site it recognizes. Thus these observations suggest a model in which PKA phosphorylation might not only increase the interaction of ERM with protein partners but also activate these partners to obtain a full transcriptional response.

At the present time, little is known about the protein partners of ERM. We have shown that this protein is able to physically interact with TBP, TAF II 40 and 60 (26) as well as the androgen receptor (20). However, one interesting candidate is the important transcriptional coactivator CBP/p300. Indeed, the androgen receptor (20). However, one interesting candidate is that this effect is more pronounced on the Coll1 promoter as compared with the E74 one (about 50 and 25% of wild-type ERM activation, respectively). An activation mediated indirectly through other proteins might thus be promoter-dependent and the activation level might reflect differences in the ability of ERM to interact with these proteins depending of the binding site it recognizes. Thus these observations suggest a model in which PKA phosphorylation might not only increase the interaction of ERM with protein partners but also activate these partners to obtain a full transcriptional response.

In conclusion, we provide evidence that the Ets transcription factor ERM and the closely related factor ETV1 are direct targets of PKA, and that activation of PKA signaling is sufficient to activate them via an unexpected mechanism, i.e. the decrease in DNA binding. To our knowledge, this is the first example of PKA-dependent phosphorylation regulating the activity of a member of the Ets family. In addition to regulation at an artificial ETS-binding site, E74 site, the PEA3-mediated activation of ER occurs at another Ets-binding site located in the collagenase 1 promoter, thus suggesting a potential regulatory mechanism for ERM functions at different promoters via the PKA-signaling pathway. Furthermore, it should be noted that, in contrast to ERM and ER81/ETV1, PEA3/1/1 is the third PEA3 group member is not directly activated by PKA. Since the three PEA3 group members display similar DNA binding specificities (23) and have been reported to be co-expressed (17, 58, 59), it is conceivable that specific promoters may therefore be targeted by different PEA3 group members depending on the kinases that phosphorylate them.

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ERM Transactivation Is Up-regulated by the Repression of DNA Binding after the PKA Phosphorylation of a Consensus Site at the Edge of the ETS Domain
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