SUMO Modification of the Ets-related Transcription Factor ERM Inhibits Its Transcriptional Activity*

Received for publication, October 1, 2004, and in revised form, March 29, 2005
Published, JBC Papers in Press, April 27, 2005, DOI 10.1074/jbc.M411250200

Cindy Degerny‡§, Didier Monte‡, Claude Beaudoin‡¶, Ellis Jaffray, Laurence Portois‡‡, Ron T. Hay‡, Yvan de Launoit‡¶§§, and Jean-Luc Baert‡

From the UMR 8117, CNRS, Université de Lille I, Institut Pasteur de Lille, Institut de Biologie de Lille, BP 447, 1 rue Calmette, 59021 Lille Cedex, France, the School of Biology, University of St. Andrews, The North Haugh, St. Andrews KY16 9ST, United Kingdom, and Laboratoire de Virologie Moléculaire, Faculté de Médecine, Université Libre de Bruxelles, CP 614, 808 route de Lennik, 1070 Brussels, Belgium

A variety of transcription factors are post-translationally modified by SUMO, a 97-residue ubiquitin-like protein bound covalently to the targeted lysine. Here we describe SUMO modification of the Ets family member ERM at positions 89, 263, 293, and 350. To investigate how SUMO modification affects the function of ERM, Ets-responsive intercellular adhesion molecule 1 (ICAM-1) and E74 reporter plasmids were employed to demonstrate that SUMO modification causes inhibition of ERM-dependent transcription without affecting the subcellular localization, stability, or DNA-binding capacity of the protein. When the adenoviral protein Gam1 or the SUMO protease SENP1 was used to inhibit the SUMO modification pathway, ERM-dependent transcription was derepressed. These results demonstrate that ERM is subject to SUMO modification and that this post-translational modification causes inhibition of transcription-enhancing activity.

Covalent modification of proteins with ubiquitin-like proteins creates new proteins with unique protein surfaces that can mediate a range of protein-protein interactions (reviewed in Ref. 1). Modification of proteins by the small ubiquitin-related modifier (SUMO) is increasingly recognized as an important regulatory mechanism that can affect the stability, subnuclear localization, and transcription-activating capacity of the protein (reviewed in Refs. 1 and 2). Several transcription factors are reported to undergo SUMO modification, including the androgen receptor (3, 4), c-Myb (5), AP-2 (6), and c-Jun and p53 (7). In addition, other transcriptional regulators, such as GRIP (8) and histone deacetylases 1 (9) and 4 (10), are also SUMO targets (the currently established targets of SUMO are reviewed in Ref. 1). Three different types of enzyme constitute the SUMO pathway: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme or carrier protein (E2), and ubiquitin-protein isopeptide ligase (E3). Although Ubc9 is the only E2 (conjugating enzyme) found in humans, multiple E3 ligases have recently been identified. SUMO modification is a reversible process, and several SUMO proteases have been identified in mammalian cells (reviewed in Ref. 1).

In mammals, the ets genes encode a large family of transcription factors, characterized by their ETS DNA-binding domain. On the basis of conservation of this and other domains, these factors have been subclassified into 13 groups (for a review, see Ref. 11). Some Ets transcription factors have been identified as targets of SUMO. For instance, Elk-1 is modified by SUMO, a modification that is reversed by signaling via the prototype extracellular signal-regulated kinase (ERK) MAPK pathway, which induces a switch from the repressed to the transcriptionally active state (12, 13). SUMO modification of Elk-1 results in the recruitment of histone deacetylase activity to promoters; this indicates the existence of an important integration point for two protein-modifying pathways in the cell, the SUMO and deacetylation pathways, which combine to promote inhibition of transcription-enhancing activity (14). SUMO has also been shown to enhance the recruitment of the Ets transcription factor Tel into repressive domains such as the PML bodies (15–17).

The three PEA3 group members (PEA3/E1AF, ER81/ETV1, and ERM/ETV5) show high conservation of their ETS domain and of the two transcriptional activation domains (reviewed in Ref. 18). These factors are involved in a number of developmental processes. For example, they play a role in the organization of the germ layers showing high proliferation and migration rates (19) and in the development of motor and sensory neurons (20, 21). These factors have also been found to be deregulated in cancer and are over-expressed in metastatic human breast cancer cells (22) and Neu-induced mouse mammary tumors (23, 24).

Post-translational modifications such as phosphorylation regulate the function of the PEA3 group members. In particular, components of the MAPK pathway have been found to...
increase the transactivation capacity of these factors. This suggests that these factors may contribute to the nuclear response to cell stimulation and also to Ras-induced transformation (25–30). The c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and cAMP-dependent protein kinase (PKA) pathways are also involved in regulating the transcription-enhancing activity of the PEA3 group members (30–32).

In the present study we have investigated whether ERM undergoes SUMO modification. We show that of the five putative SUMO modification sites of this protein, the first four can be conjugated to SUMO. We present the results of functional studies showing that this post-translational modification can inhibit ERM transcription-enhancing activity.

MATERIALS AND METHODS

Yeast Two-hybrid Screen—The yeast two-hybrid screening was performed with the MATCHMAKER two-hybrid system as recommended by the manufacturer (Clontech). A DNA fragment encoding amino acids 72–370 of ERM was generated by PCR amplification and subcloned in-frame with the LexA DNA-binding domain contained in the pLexA-ERM expression vector pLexA (Clontech) at the EcoRI restriction site. The resulting plasmid was transformed using a standard polyethylene glycol/LiAc-mediated transformation procedure into the EGY48 strain of yeast. An oligo(dT)-primed mammary gland cDNA library (Clontech) constructed in the yeast galactose-inducible expression plasmid pB42AD was introduced into the EGY48 yeast strain harboring the pLexA-ERM construct. The resulting co-transformants were grown at 30 °C in the absence of histidine, tryptophan, and uracil (selective minimal media). A total of 3 × 10^6 clones were screened for the capacity to grow on selective minimal media. Colonies growing in the absence of leucine and scoring positive for β-galactosidase activity by the filter assay method were directly recovered from the plate and grown in selective media lacking leucine. cDNA inserts from the positive yeast colonies were electrotransformed into KC8 Escherichia coli tryptophan auxotrophic bacterial cells. Transformants were grown on M9 (–Trp, –Amp) minimal medium to select for plasmids containing the pB42AD vector. The cDNA inserts were then sequenced by automated sequence analysis (ABI sequence analyzer).

Plasmid Constructs—The full-length and amino-terminal truncated pSG5-ERM expression vectors and the pSV-HA-ERM vector have been described elsewhere (32). The 5′-FLAG-tagged ERM vector (pSV-FLAGERM) was constructed using the ERM cDNA and the pSV plasmid used previously (33) (details are available upon request). The various ERM mutants (Lys to Arg and Glu to Ala) were generated with a QuikChange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing. His6-SUMO conjugates were purified by metal-chelate affinity chromatography as described previously (37). Briefly, cells plated in 6-well plates were washed twice in ice-cold phosphate-buffered saline and lysed in 400 μl of lysis buffer (6 M guanidine hydrochloride, 0.1 M NaH2PO4/Na2HPO4, pH 8.0, 0.01 M Tris-HCl, pH 8.0)/35-mm well. The lysates were centrifuged at 100,000 × g for 90 min at 4 °C. Each sample was mixed with 20 μl of packed Ni2+–nitrilotriacetic acid beads (Qiagen) and then incubated for 1 h at room temperature. The beads were then washed twice with lysis buffer, three times with 8 M urea, 0.1 M NaH2PO4/LiH2PO4, pH 6.4, and once with phosphate-buffered saline before being resuspended in Laemmli gel loading buffer and analyzed by SDS-PAGE. Proteins were then electrophotographically transferred to nitrocellulose membranes, and His-SUMO-ERM conjugates were detected by Western blotting using a rabbit polyclonal anti-ERM antibody as described above.

In Vitro SUMO Modification Reaction—[35S]Metionine-labeled substrates for SUMO modification reactions were generated by in vitro transcription/translation in wheat germ extract according to the manufacturer’s instructions (Promega). In vitro modification was carried out with purified recombinant products as described (34). Reaction products were fractionated by SDS-PAGE and detected by phosphorimaging.

Electrophoretic Mobility Assay (EMSA)—The protein ERM was produced and sumoylated in vitro as described above. A portion of the reaction mixture was mixed with 1 ng of the 32P-labeled E74 probe (sense strand, 5′-AGCTGAATAACCCAGGATCTCAT-3′) in the presence of 25 mM Hepes, pH 7.9, 25 mM KCl, 2 mM MgCl2, 1 mM EGTA, 0.05% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 1 μg of poly(dIdC). The mixture was incubated for 1 h at room temperature and loaded onto a 6% polyacrylamide gel. The gel was run at 4 °C in 0.5× Tris borate-EDTA buffer at 180 V (32).

Immunofluorescence Studies—RK13 cells plated on coverslip slides were transfected with the different FLAG-tagged ERM plasmids. Twenty-four h later the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then incubated for 2 h with 1:1000 diluted anti-FLAG antibody. This was followed by incubation for 1 h with 1:1000 diluted goat anti-rabbit Alexa 488 antibody.

RESULTS

SUMO Modification of ERM—To identify ERM-interacting proteins, we screened a human mammary gland cDNA library in the yeast two-hybrid system using a nontransactivating portion of the ERM molecule (amino acids 72–370) as bait. Twelve isolated clones were found to encode the human Ubc9. GST pull-down assays were used to obtain direct evidence for ERM binding to Ubc9 (data not shown), thus confirming Ubc9 as a novel ERM-interacting protein.

Because Ubc9 is the only known SUMO-conjugating enzyme (38) and because ERM displays five minimal consensus sumoylation motifs (ψKKXE; ψi, large hydrophobic residue (39)), we sought to determine whether ERM is a sumoylation target using in vitro and in vivo approaches. To show directly that
ERM is a SUMO modification substrate, \(^{35}S\)-labeled ERM was generated by in vitro transcription/translation and incubated with purified components required for SUMO modification (34). As illustrated in Fig. 1A, analysis of the products indicates that a large proportion of the ERM was converted by SUMO-1, SUMO-2, or SUMO-3 to more slowly migrating forms in a reaction requiring the SUMO-activating enzyme E1 and the E2 enzyme Ubc9. To establish that ERM is modified by SUMO in vivo, we co-transfected RK13 cells with expression plasmids for ERM and His\(_{16}\)-tagged SUMO-1, -2, or -3. Cells were lysed under conditions preserving SUMO modification, and ERM proteins were identified by Western blotting with an anti-ERM antibody. In the absence of SUMO, we observed a major 70-kDa protein species, corresponding to unmodified ERM. Higher molecular weight species were detected only when SUMO-1, SUMO-2, or SUMO-3 was present (Fig. 1B). Under these conditions, the presence of multiple high molecular weight species suggests that multiple SUMO modifications take place on ERM. Similar in vivo experiments were performed on other cell lines, such as COS-7. Again, the upper bands also appeared in the presence of each SUMO and were much pronounced in the presence of SUMO-2 (Fig. 1B). To prove that these upper bands corresponded to SUMO-modified forms of ERM, COS-7 cells transfected with ERM and His-SUMO-2 were lysed under highly denaturing conditions, and SUMO-modified proteins were isolated on nickel-agarose (Ni\(^{2+}\)). Nickel-nitrilotriacetic acid-bound proteins were then eluted and analyzed by Western blot with anti-ERM antibody. In the absence of SUMO, we observed a major 70-kDa protein gives a two-band pattern because of internal methionine initiation. The upper bands were isolated on nickel-agarose (Ni\(^{2+}\))—as in Fig. 1C—were then analyzed by SDS-PAGE, and ERM was then detected by Western blotting with an anti-ERM antibody.

**SUMO Modification Sites on ERM**—ERM contains five copies of the \(\psi\)KXE sequence required for modification by SUMO (Fig. 2A). To locate sites of SUMO modification on the ERM protein, we generated amino-terminal deletion mutants of ERM and tested their ability to be SUMO-modified in vitro by co-transfection with His-SUMO-2 (as in Fig. 1C). Although wild-type ERM was modified by SUMO at multiple sites, ERM (276–510) was modified at only one site and ERM (354–510) did not appear to be SUMO-modified (Fig. 2A). These data suggest that residues Lys\(^{89}\) and Lys\(^{263}\) are likely targets of SUMO modification, along with either Lys\(^{263}\) or Lys\(^{350}\). It is unlikely that Lys\(^{468}\), although in a sequence matching perfectly the consensus sumoylation motif, is a site of SUMO modification.

To determine precisely the sites of SUMO modification, we therefore replaced each potential acceptor lysine, separately or in combination, with an arginine unable to act as a SUMO acceptor. COS-7 cells were then co-transfected with a plasmid coding for wild-type or mutated ERM together with the SUMO-2 expression vector. ERM was revealed by Western blotting. Fig. 2B shows that the single mutation of Lys\(^{468}\) (KR\(^{5}\)) has no significant effect on ERM SUMO modification. A single mutation of Lys\(^{263}\) (Fig. 2B, KR\(^{1}\)), Lys\(^{263}\) (KR\(^{2}\)), or Lys\(^{350}\) (KR\(^{3}\)) reduced the intensity of at least some SUMO-modified ERM species, and a more pronounced effect was observed for the Lys\(^{283}\) mutation (KR\(^{4}\)). When Lys\(^{263}\) and Lys\(^{293}\) (KR\(^{3}\)) were mutated in combination, the more slowly migrating SUMO-conjugated species of ERM were no longer observed, but the major 110–120 kDa was still clearly detected. Further mutation of Lys\(^{468}\) (Fig. 2B, KR\(^{4}\)) had little effect on the SUMO modification pattern, whereas mutation of Lys\(^{468}\) in combination with KR\(^{4}\) (KR\(^{5}\)) caused the amount of the major SUMO-modified species to decrease markedly. This modified form was practically undetectable when Lys\(^{89}\), Lys\(^{263}\), Lys\(^{293}\), and Lys\(^{350}\) were mutated simultaneously (Fig. 2B, KR\(^{23}\)), and no further change was observed with ERM mutated at all five consensus sumoylation sites (KR\(^{23}\)). Simultaneous mutation at all of the five consensus sites completely prevented SUMO modification of ERM in vitro (Fig. 2C). These findings suggest that SUMO modification occurs only at consensus sites and that, of the five consensus sites identified in ERM, the motif containing Lys\(^{468}\) in the carboxyl-terminal part of ERM is the only one that is not SUMO-modified in vitro.

**ERM is SUMO-modified in Response to Stress in Vivo**—Because cell stresses such as heat shock or oxidative stress induce SUMO conjugation (40), we investigated the ability of endogenous SUMO to modify ERM in vivo upon exposure of cells to hydrogen peroxide (H\(_{2}\)O\(_{2}\)). For this purpose, COS-7 cells were transfected with plasmids coding for wild-type or KR\(^{23}\)FLAG-ERM, and transfected cells were treated or not with H\(_{2}\)O\(_{2}\). The cell extracts were then subjected to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitates were analyzed by Western blot with antibodies.
against ERM or SUMO. With the anti-ERM antibody, unmodified ERM was detected independently on H₂O₂ treatment. In contrast, more slowly migrating ERM species were observed specifically in the H₂O₂-treated cells. These H₂O₂-induced more slowly migrating ERM species were not observed with the FLAG-ERM KR12345 mutant (Fig. 3); their appearance was thus dependent on the presence of the sumoylation sites. To determine which form of SUMO was conjugated to ERM, extracts from H₂O₂-treated cells immunoprecipitated with the anti-FLAG antibody were analyzed by Western blotting using either an anti-SUMO-1 or an anti-SUMO-2/3 antibody. The more slowly migrating forms of ERM were detected with the anti-SUMO-2/3 antibody but not with the anti-SUMO-1 antibody. These forms were absent when ERM KR12345 was used. This indicates that endogenous SUMO-2 or/and -3 are responsible for H₂O₂-induced ERM sumoylation in vivo.

SUMO Sites in ERM Correlate with Inhibition of Transcription-enhancing Activity—To investigate the effect of SUMO modification on ERM transcription-enhancing activity, we analyzed the ability of wild-type and mutant forms of the protein to activate ERM-dependent reporter genes. In experiments with the human Ets-responsive ICAM-1 minimal promoter (35), the wild-type ERM caused a 20-fold increase in transactivation (Fig. 4A, WT). ERM singly mutated at any one of the five consensus sumoylation sites showed the same or nearly the same transactivation power as wild-type ERM (Fig. 4A, left panel). Triply mutated ERM proteins KR123 and KR234, however, showed an ~3-fold higher transactivation capacity. No further increase was observed with the KR1234 and KR12345 ERM mutants (Fig. 4A). The second luciferase reporter vector used contains three E74 Ets-binding sites cloned upstream from the minimal thymidine kinase promoter. Although ERM specifically interacts with the E74 binding site, it cannot activate the E74 reporter construct without stimulation by post-translational modification such as phosphorylation (32). As shown in Fig. 4A (right panel), RK13 cells co-transfected with

**Fig. 2. Identification of the SUMO modification sites of ERM.** A, SUMO modification of ERM after various deletions. Upper panel, schematic representation of the positions of the five putative SUMO modification sites (ΔKXE) within ERM and of the deletion mutants used. The main functional ERM domains are indicated (ETS, ETS domain; TAD, main transactivation domain). Lower panel, COS-7 cells were transfected with plasmids expressing ERM deletion mutants, and SUMO modification was assessed by Western blotting with an anti-ERM antibody on the crude extracts (Crude) and after purification on an Ni²⁺ column. The positions of unmodified (arrow) and SUMO-modified (star) ERM forms are indicated. B, in vivo SUMO modification of ERM after the replacement of lysine with arginine (KR) in the putative SUMO modification sites. SUMO modification of ERM mutants in COS-7 cells was determined by Western blotting with an anti-ERM antibody as described for Fig. 1B. As a control, cells were transfected with a plasmid expressing wild-type ERM (WT) in the absence or the presence of a co-transfecting plasmid expressing SUMO-2. Superior numbers 1, 2, 3, 4, and 5 correspond, respectively, to Lys⁸⁹, Lys²⁶³, Lys²⁹³, Lys³⁵⁰, and Lys⁴⁶⁸. C, in vitro SUMO modification of wild-type ERM and of ERM mutated at all five putative SUMO modification sites (KR12345). The assay was performed as described for Fig. 1A.
the E74 reporter and a plasmid expressing either the wild-type or a singly mutated ERM showed similar, near basal level luciferase activity. When a multiply mutated ERM was used, transactivation increased about 4-fold (KR<sup>234</sup>) to 8-fold (KR<sup>123</sup>, KR<sup>234</sup> and KR<sup>12345</sup>) (Fig. 4A). As assessed by immunofluorescence, the sumoylatable (Fig. 4B, WT) and the nonsumoylatable (KR<sup>12345</sup>) ERM forms similarly localized in the nucleus in a diffuse manner. Localization of ERM was not affected by overexpression of SUMO-2. Altogether, these results clearly show that the nonsumoylatable ERM mutant has an enhanced transcriptional capacity and that it is not due to change in intracellular localization.

We then investigated whether change in the transcriptional activity of the sumoylation defective ERM mutant is due to variation of protein stability as previously reported for other proteins (41, 42). We thus examined the steady-state levels of wild-type and nonsumoylatable ERM following inhibition of protein synthesis. COS-7 cells transfected with ERM plasmid were treated from 20 to 80 min with the protein synthesis inhibitor cycloheximide and analyzed by Western blotting. In contrast to actin, which is not influenced by cycloheximide treatment, wild-type ERM is relatively unstable with a half-life of about 40 min; this relatively weak stability is also found in the nonsumoylatable KR<sup>12345</sup> ERM mutant (Fig. 5A). Similar experiments were also performed in cells transfected with wild-type ERM and SUMO-2 to examine the stability of the SUMO-modified forms of ERM. As illustrated in Fig. 5A, the SUMO-modified forms showed similar stability as compared with un-

![Fig. 3. SUMO modification of ERM is induced by H<sub>2</sub>O<sub>2</sub> stress. COS-7 cells were transfected for 24 h with a plasmid expressing FLAG-ERM (left panels) or FLAG-ERM KR<sup>2345</sup> (right panels). The cells were then treated or not with H<sub>2</sub>O<sub>2</sub>. Upper panels, Western blots obtained from crude protein extracts with anti-ERM antibody as described for Fig. 1B. Lower panels, following ERM immunoprecipitation (IP) with an anti-FLAG antibody, protein extracts were subjected to Western blotting (immunoblot (IB)) with anti-SUMO-1 or anti-SUMO-2/3 antibody. *, indicates immunoprecipitated ERM-like forms recognized by anti-SUMO antibodies.

![Fig. 4. Lysine-to-arginine mutations at the ERM SUMO modification sites increase transcription. A, effect of lysine-to-arginine mutations. RK13 cells were transiently transfected for 24 h with ICAM-1-Luc (ICAM) or 3xE74-TK-Luc (E74) reporter plasmid in the presence of pSV-ERM wild-type (WT) and lysine-to-arginine (KR) mutants in the SUMO modification sites. The data presented here are representative of four experiments with two different dishes per experiment and are expressed as means ± S.E. The activity of cells transfected with the reporter plasmid and the empty pSV expression vector (−) is arbitrarily set at 1. B, subcellular distribution of ERM. RK13 cells were transfected with FLAG-tagged ERM (WT) or FLAG-tagged ERM-KR<sup>2345</sup> (KR<sup>2345</sup>) in the presence or absence of SUMO-2. Expressed proteins were detected by immunofluorescence microscopy. Hoechst staining shows nuclei.

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modified wild-type ERM or the KR$_{12345}$ ERM mutant. These results indicate that neither mutation of the sumoylation sites nor sumoylation of ERM significantly affects ERM stability.

We also tested whether the DNA-binding capacity of the nonsumoylatable ERM form varies from that of wild-type protein. Using EMSA with nuclear extracts of COS-7 cells overexpressing ERM or its nonsumoylatable mutant KR$_{12345}$ we showed that the specific DNA binding was not affected by the mutation of the sumoylation sites (data not shown). We had shown previously, however, that in vitro only a small proportion of the overexpressed ERM protein is SUMO-modified, and the influence of sumoylation on ERM DNA-binding capacity was thus difficult to evaluate in these conditions (Fig. 2B). To circumvent that, we subjected ERM produced by in vitro translation to in vitro SUMO modification and then tested its DNA-binding ability. Under these conditions, ERM was almost fully SUMO-modified (Fig. 5B, SDS-PAGE), and gel shift analysis with an E74 probe specifically recognized by ERM (32) revealed a lower mobility of SUMO-modified ERM-DNA complex (Fig. 5B, EMSA). However, similar band intensities were observed for the SUMO-modified ERM-DNA and the unmodified ERM-DNA complexes (Fig. 5B, EMSA; compare lanes 2 and 3). This indicates that difference in transcriptional activity between wild-type and sumoylation-defective ERM is independent of DNA-binding ability.

**SUMOylation Has a Negative Effect on Transcription Regulation by ERM**—We thus have shown that SUMO modification of ERM did not affect the subcellular localization, the protein stability, and the DNA-binding activity of ERM, whereas mutation of the lysine within the SUMO sites induced transcriptional activation. However, in addition to sumoylation, the lysine residue can be modified by a number of post-translational modifications including methylation, ubiquitination, and acetylation. To ensure that enhanced transcriptional activity of ERM mutated at all of the lysine acceptor sites (KR$_{12345}$) is specifically due to defective SUMO modification, we tested the activity of the mutant protein obtained by changing the glutamic acid of each consensus sumoylation site to alanine, without mutating the lysine residue of the site. Such mutations are reported to disrupt SUMO transfer by affecting the interaction of the substrate with the SUMO-conjugating enzyme Ubc9 (43).

In RK13 cells, the effect on transcription of ERM mutated at all five glutamic acid residues (EA$_{12345}$) was similar to that of the KR$_{12345}$ ERM mutant in both the ICAM-1 and E74 reporter systems (Fig. 6A). Similar results were also obtained in HeLa cells. As expected, the EA$_{12345}$ ERM mutant, like the KR$_{12345}$ ERM mutant, could not be modified by SUMO, in contrast to wild-type ERM (Fig. 6B), thus confirming that the loss of SUMO modification correlates with enhanced transcriptional activation of ERM.

The data obtained strongly suggested that sumoylation inhibits the transcriptional activity of ERM. We thus determined whether decreased sumoylation reverses the inhibition. In reporter gene assays, we used two proteins known to interfere with the SUMO conjugation pathway: the adenoviral protein Gam1, which inhibits the SUMO pathway by inducing the degradation of SUMO-activating (E1) and -conjugating (E2) enzymes (44); and SENP1, a SUMO-specific protease involved in desumoylation (36). Neither protein had any effect on basal transcription from the ICAM-1 and E74 reporter plasmids (Fig. 7). In the E74 reporter system, both Gam1 and SENP1 caused a major (~6-fold) increase in the activity of wild-type ERM. This increase was dependent on the presence of the sumoylation sites, since neither Gam1 nor SENP1 altered the transcriptional activity of the KR$_{12345}$ ERM mutant (Fig. 7). In reporter system based on the ICAM-1, results were similar. Both interfering proteins increased transactivation by wild-type ERM, and KR$_{12345}$-induced activity remained unaltered in the presence of these molecules. Moreover, the enhancement of ERM transactivation capacity was due to the ability of these two proteins to interfere with the SUMO pathway, as the transcriptional activity of ERM was only marginally changed in coexpression of inactive versions of Gam1 and SENP1 (Gam1mut, Gam1L258,265A; SENP1mut, SENP1R630L,K631M) (Fig. 7). These data therefore demonstrate that the transcriptional activity of ERM is inhibited by the SUMO pathway.

**DISCUSSION**

ERM transcription-enhancing activity is regulated by post-translational modifications such as phosphorylation via the MAPK and cAMP-dependent protein kinase pathways (25, 32). Here we show that ERM interacts with the SUMO-conjugating enzyme Ubc9 and is modified by SUMO. We further show that
SUMO modification of this Ets transcription factor affects its ability to activate transcription.

We have demonstrated that four of the five lysines located in optimal sumoylation consensus motifs (\(\phi\)KXE) of ERM were modified by SUMO both \textit{in vitro} and \textit{in vivo}. When either the acceptor lysines or the glutamic acid residues of these sites are mutated, SUMO modification of the mutated ERM does not occur. These SUMO acceptor sites are conserved in human, mouse, chicken, and zebrafish ERM homologues. This suggests that SUMO modification of the ERM transcription factor plays an important role in vertebrates. These sites are also perfectly conserved in the two other PEA3 group members, ETV1 and PEA3. We have observed that both of these also undergo \textit{in vivo} SUMO modification (data not shown). Although SUMO-2 seems to conjugate preferentially with ERM, the SUMO modification profiles obtained for ERM in RK13 cells are similar for SUMO-1, -2, and 3. Unlike SUMO-2, SUMO-1 cannot form poly-SUMO chains (34). It is likely that the more slowly migrating SUMO-modified forms observed reflect monosumoylation of ERM at multiple sites. SUMO modification at a single acceptor site also occurs. The corresponding modified protein must be the major ERM species (about 110–120 kDa), because this SUMO-modified ERM form is the only one remaining when three of the four SUMO conjugation sites are mutated. The observed molecular mass of this modified ERM form is, however, much higher as expected (about 85 kDa; ERM is about 70 kDa, and His-SUMO is about 15 kDa). Although surprising, this phenomenon is common for SUMO-modified molecules and is probably due to an altered conformation of the modified proteins.

Selective addition of SUMO-1, -2, and -3 (SUMO-2 and -3...
being highly homologous) to protein targets has not been studied extensively. Studies have indicated that SUMO-1 can conjugate with proteins via mechanisms similar to the conjugation mechanism of SUMO-2 and SUMO-3, but it has been suggested that SUMO-1 on the one hand and SUMO-2/3 on the other show different substrate specificities (40, 45). When ectopically expressed, all three SUMO isoforms have the capacity to become conjugated to ERM, although SUMO-2 conjugates predominate in COS-7 cells. Interestingly, when ERM was expressed in COS-7 cells treated with H$_2$O$_2$, only the covalent attachment of endogenous SUMO-2 and/or -3 was visualized. This is in agreement with the observation that the incorporation of SUMO-2/-3 into conjugates increases in response to oxidative stress (40). Whether this actually reflects a preferential modification by SUMO-2 remains, however, to be determined.

SUMO modification has been reported for several proteins, and most of them exert their main function within the nucleus as transcription factors or transcriptional co-activators or corepressors (for review, see Ref. 1). Here, we show that the Ets transcription factor, ERM, is submitted to SUMO modification and that this post-translational modification negatively affects its transcription-activating function, because SUMO modification-deficient mutant ERM proteins display a greater capacity to activate transcription. Moreover, two molecules known to down-regulate SUMO modification by distinct mechanisms (Gam1 and SENP1) enhanced ERM transcriptional activity, whereas they induced only a slight variation of the KR$^{12345}$ transcriptional activity. Altogether, these data argue for an important role of SUMO modification on ERM transcriptional activity. A correlation between SUMO modification and inhibition of transcription-regulating activity has previously been observed for other transcription factors such as Elk-1, Sp3, and c-Myb (for review, see Ref. 1). Most of these SUMO-modified proteins possess fewer SUMO acceptor sites than ERM, which has four of them. This relatively high number of SUMO sites may be responsible for fine regulation through sumoylation. It appears that SUMO modification of all sites is not necessarily required to block the full transcriptional activity of ERM. Although mutation of a single sumoylation site does not significantly increase the action of ERM on the promoters used, mutation of three of the four sites (KR$^{123}$ and KR$^{234}$) is sufficient to obtain the full transcriptional de-repression observed when ERM is mutated at all five consensus sites (KR$^{12345}$). This applies, however, only to the ICAM-1 promoter context, because on the E74 promoter, ERM KR$^{123}$ displays a weaker transcription-enhancing effect than ERM KR$^{234}$. This suggests that according to the promoter context, the repressive effect of sumoylation may vary in function of the number and identity of SUMO-modified sites.

As reported for other SUMO-modified proteins, such as the Ets protein Elk-1 (13), only a small proportion of total ERM protein is SUMO-conjugated. However, mutation of the sumoylation sites on ERM or inhibition of the sumoylation process has a significant effect on the ERM transcription-enhancing activity. This is probably because SUMO modification is a highly dynamic process and this modification, although required to initiate transcriptional inhibition, is not necessary to maintain this inhibition. The molecular mechanism associated to SUMO on inhibition of transcription-enhancing activity is still unclear. Here, we show that this inhibition is not related to changes in the stability or DNA-binding activity of ERM. It might be that repression could be linked to SUMO itself, because SUMO possesses an intrinsic repressive ability when fused to the DNA binding of Gal4 (1). Such a repressive mechanism could involve histone deacetylase recruitment, as demonstrated previously for p300 (46) and Elk-1 (14). It is, however, possible that SUMO interferes with the recruitment of co-factors by ERM by decreasing the interaction with transcriptional elements required for transcriptional activity. In fact, SUMO-induced transcriptional activity of ERM could be regulated by CBP, which is a co-activator of the PEA3 group members (47) and a target of SUMO modification. Indeed, sumoylation could disfavor the recruitment of CBP by ERM, thus reducing the ERM transcriptional activity, and reduced ERM activity could be enhanced by the SUMO-dependent repression of CBP activity by recruitment of HDAC6 (46).

It has been shown that SUMO conjugation targets proteins to different cellular localizations. We observed no difference in nuclear localization between wild-type ERM and its SUMO conjugation-deficient mutant. Yet, we cannot exclude the possibility that SUMO modification of ERM might direct the protein, in a subtle manner not currently observed, into some particular nuclear domains or might affect the nuclear import/export shuttling of this transcription factor, as shown previously for the Ets proteins Tel and Elk-1 (17, 48). More experiments are needed to specify the mechanisms by which SUMO controls the activity of ERM.

Acknowledgment—We are grateful to Yves Rouillé for helpful discussions.

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J. Biol. Chem. 2005, 280:24330-24338.
doi: 10.1074/jbc.M411250200 originally published online April 27, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M411250200

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