Down-Regulation of c-Fos/c-Jun AP-1 Dimer Activity by Sumoylation

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The inducible transcriptional complex AP-1, composed of c-Fos and c-Jun proteins, is crucial for cell adaptation to many environmental changes. While its mechanisms of activation have been extensively studied, how its activity is restrained is poorly understood. We report here that lysine 265 of c-Fos is conjugated by the peptidic posttranslational modifiers SUMO-1, SUMO-2, and SUMO-3 and that c-Jun can be sumoylated on lysine 257 as well as on the previously described lysine 229. Sumoylation of c-Fos preferentially occurs in the context of c-Jun/c-Fos heterodimers. Using nonsumoylatable mutants of c-Fos and c-Jun as well as a chimeric protein mimicking sumoylated c-Fos, we show that sumoylation entails lower AP-1 transactivation activity. Interestingly, single sumoylation at any of the three acceptor sites of the c-Fos/c-Jun dimer is sufficient to substantially reduce transcription activation. The lower activity of sumoylated c-Fos is not due to inhibition of protein entry into the nucleus, accelerated turnover, and intrinsic inability to dimerize or to bind to DNA. Instead, cell fractionation experiments suggest that decreased transcriptional activity of sumoylated c-Fos is associated with specific intranuclear distribution. Interestingly, the phosphorylation of threonine 232 observed upon expression of oncogenically activated Ha-Ras is known to superactivate c-Fos transcriptional activity. We show here that it also inhibits c-Fos sumoylation, revealing a functional antagonism between two posttranslational modifications, each occurring within a different moiety of a bipartite transactivation domain of c-Fos. Finally we report that the sumoylation of c-Fos is a dynamic process that can be reversed via multiple mechanisms. This supports the idea that this modification does not constitute a final inactivation step that necessarily precedes protein degradation.

The AP-1 transcription complex is a family of dimeric transcription factors that bind to the TPA-responsive element (TRE)/AP-1 DNA motifs and related sequences (14). Via activation and repression of a broad array of genes, AP-1 is a regulator of major physiological processes such as cell proliferation, differentiation, organogenesis, apoptosis, and response to stress (57). It is also a necessary effector in a wide variety of pathological situations, including tumorigenesis (26, 63). Indeed, certain of its components can manifest oncogenic and/or tumor suppressor activity depending on the cell context (19).

c-Fos and c-Jun are the best-studied AP-1 components. They share a number of homologous domains, including adjacent basic and leucine zipper motifs, necessary for binding to DNA and dimerization, respectively. In contrast to c-Fos, c-Jun can homodimerize. However, heterodimerization with partners, such as c-Fos, is favored (14). c-Fos can act positively on transcription of a variety of genes, including collagenase I (*MMPI*) (14, 54). As for many other transcription factors, one essential mechanism by which c-Fos- and c-Jun-containing AP-1 dimers activate transcription involves direct contacts with coactivators, such as the CBP/p300 acetyltransferases (5, 6),

and constituents of the basal transcription machinery, such as the TATA-binding protein (TBP) (21, 33).

The *c-fos* and *c-jun* genes are expressed constitutively in certain tissues. They are, however, considered immediate-early genes because their expression is usually low but inducible rapidly and, most often, transiently in response to a wide array of stimuli to allow cells to adapt to environmental changes (26, 66). To avoid the deleterious effects of improper expression, both *c-fos* and *c-jun* are subjected to numerous transcriptional and posttranscriptional regulations (30, 44). At the protein level, their activation mechanism, primarily by phosphorylation (30) (also see text below), is relatively well studied, whereas those of inactivation remain ill-defined.

An essential repression mechanism is protein destruction, c-Jun and c-Fos being rapidly degraded by the proteasome (51). Notably, the bulk of c-Fos can be broken down independently of the ubiquitylation of the protein (8) through complex, regulated mechanisms (3, 20), whereas only ubiquitin-dependent degradation of c-Jun has been reported thus far in vivo (62). However, other processes are likely to precede protein disappearance. In a search to identify posttranslational modifications possibly repressing its activity, we noted that c-Fos contains a ψ KXE (where ψ is a large hydrophobic residue, K the conjugated lysine, E glutamic acid, and X any amino acid) consensus motif (45) for conjugation by SUMO, a peptidic posttranslational modifier structurally related to ubiquitin and conjugated on acceptor lysines.

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Three SUMO isoforms (SUMO-1, SUMO-2, and SUMO-3) are expressed in mammalian cells, SUMO-1 being the most extensively studied. The SUMO pathway resembles that of ubiquitin (24, 27, 36). It utilizes a single heterodimeric E1 SUMO-activating enzyme, Sae1/Sae2, and one E2 SUMO-conjugating enzyme, Ubc9. Although SUMO E1 and E2 are usually sufficient for sumoylation of substrates in vitro, a third component, E3, is also likely to be used in vivo for substrate selection and to ensure the specificity of reaction. Among the few characterized E3s are certain PIAS proteins (24, 27, 36). SUMO modifies a variety of predominantly nuclear proteins and is involved in many processes as diverse as intracellular distribution, stability, enzymatic activity, and protein-protein or protein-DNA interactions (24, 27, 36, 55).

Among the sumoylated proteins, a growing number of transcription factors and repressors, some of which are involved in tumorigenesis (36), have been described. While certain of these transcription factors are activated upon sumoylation, most of them show decreased transactivation activity when modified by SUMO via mechanisms that are essentially awaiting elucidation (24, 55). One striking feature of SUMO modification is that the biological consequences of conjugation are not proportional to the usually small fraction of modified substrate that is detected. Importantly, SUMO conjugation may alter the long-term fate of modified proteins, even after deconjugation of SUMO, and there is evidence that an unmodified protein with a history of SUMO modification may have different properties from a protein that has never been modified (reviewed and discussed in detail in reference 25).

c-Jun has already been reported to be a target for SUMO-1 on lysine 229, which reduces its transactivation activity, albeit modestly (35). Moreover, in the presence of the PIAS SUMO E3s, another minor unidentified lysine is also modified (53). Whether this sumoylation event also alters c-Jun function is, however, not known. As already mentioned, c-Fos contains a perfect sumoylation consensus site involving K265 (LKAEE) but also two related ones involving K127 (GKLE) and K191 (EKLE). We therefore asked whether it could also be sumoylated in vivo, possibly in a regulated manner, and how this posttranslational modification could affect its activity. As c-Fos does not act alone but rather when heterodimerized with other AP-1 proteins, we investigated its modification by SUMO in the context of c-Fos/c-Jun heterodimers since c-Jun is its major heterodimerization partner in many physiological situations.

MATERIALS AND METHODS

Plasmids, cloning, and mutagenesis. Mutagenesis and clonings were carried out using standard PCR-based techniques. All details on expression plasmids are available upon request. The parental c-Fos and c-Jun expression plasmids (3, 35) and leucine zipper and DNA binding mutants (8, 20) have been described. Constitutive expression vectors were derived from pcDNA3 (Invitrogen) whereas serum-inducible ones were based on the PM302 vector (3). The expression plasmids for His₆-SUMO-1, SUMO-2, and SUMO-3 are gifts from R. Hay. pEGFP-C1 is from Clontech. The pColl-517/+63-Luc-pGL3 and pColl-517/+63mTRE-LucpGL3 reporter plasmid carries a firefly luciferase gene under the control of the AP-1-responsive mouse collagenase promoter (gift from H. van Dam). The plasmid expressing Ha-RasG12V has been described (65).

Cell culture, transfection conditions, luciferase assays, and immunoblotting experiments. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. *c-fos*^{-/-} f10 cells have been described (10). HeLa cells are available from the American Type Culture Collection. HeLa-SUMO-1, -SUMO-2, and -SUMO-3 cell lines are kind gifts from R. Hay. For in vivo

sumoylation assays, 3.5×10^6 HeLa cells were cotransfected with 30 μ g of His₆-SUMO and 30 μ g wild-type or mutant c-Fos expression vectors using the calcium phosphate coprecipitation procedure. Cells were harvested 36 h later for analysis.

For transactivation assays, 0.4×10^6 HeLa cells were transfected with 200 ng of the pColl-517/+63-Luc-pGL3 reporter plasmid, 500 ng of pEGFP-C1 plasmid as a normalization control, and different quantities of c-Fos and c-Jun expression plasmids using Fugene (Roche). Luciferase activity was assayed 24 h later using the Reportlight kit from Biowhitaker. Control experiments were carried out with the pColl-517/+63mTRE-LucpGL3 reporter plasmid in which the AP-1 site is mutated to make sure that transcription of mutated and chimeric c-Fos proteins is dependent on the AP-1 site contained in the collagenase I promoter (not shown). When expression vectors for proteins with different half-lives were compared, plasmid concentrations were adjusted for expression of equal amounts of the proteins studied, as assayed by immunoblotting.

For serum synchronization experiments, mouse cells were serum starved for 36 h to arrest them in G₀ and stimulated by fresh medium containing 20% fetal calf serum. In the case of HeLa cells, a 36-h impoverishment of the culture medium was sufficient to permit c-Fos induction by addition of fresh medium containing 20% serum. Immunodetection of c-Fos was carried out as previously described (3) using the sc52 anti-c-Fos antibody (Santa Cruz). Two different sc52 batches were used, which detected different background proteins, indicated by bb in the figures. MEK1 was inhibited by addition of 10 μ M UO126 (Cell Signaling). Inhibition of JNK, p38/SAPK, and phosphatidylinositol 3-kinase was obtained by addition of 10 μ M SP600125 (Calbiochem), SB203580 (Alexis), and LY294002 (Alexis), respectively. As inhibitors were diluted in dimethyl sulfoxide, control experiments with only dimethyl sulfoxide were also carried out (not shown). Erk1/2 were detected in immunoblotting experiments as above using the p42/44 mitogen-activated protein kinase antibody and phospho-Erk1/2 with the anti-phospho-p44/44 mitogen-activated protein kinase (Thr202/Tyr204) antibody from Cell Signaling. Inhibition of JNK, p38/SAPK, and phosphatidylinositol 3-kinase was tested by immunoblotting using antisera against phospho-c-Jun (Cell Signaling, catalog no. 92615), phospho-MAPKAPK2 (Cell Signaling, catalog no. 38415), and phospho-AKT (Cell Signaling, catalog no. 4058P), respectively. The anti-phospho-T232 c-Fos antiserum (catalog no. 44-280G) was from BioSource. Nonspecific antibodies contained in this antiserum were exhausted by incubation with immunoblotting membranes of c-Fos-nonexpressing HeLa cell extracts before use. The anti-Flag monoclonal antibody was from Sigma.

SUMO conjugate purification and analysis. His₆-tagged proteins were affinity chromatography purified as described (46). For immunoblotting experiments, cells were lysed in 1% sodium dodecyl sulfate (SDS)-containing sample buffer. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto polyvinylidene difluoride membranes. Immunodetection of c-Fos species was carried out as described above.

Gel shift assay. Proteins were in vitro translated using the TNT kit (Promega). Electrophoretic mobility shift assays were conducted as described by Nissen et al. (39) with 1 μ l of in vitro-translated proteins with a ³²P-end-labeled oligonucleotide containing an AP-1 site (TTCCGGTCTGACTCATCAAGCG) in a buffer made of 10 mM HEPES, pH 7.9, 20 mM NaCl, 4 mM MgCl₂, 1 mM EDTA, 2 mM spermidine, 5% glycerol, and 10 mM dithiothreitol. Competition experiments were conducted with the nonradioactive oligonucleotide or with an oligonucleotide containing a randomized AP-1 sequence (TTCCGGAGTATCCTCAAGCG).

In vitro sumoylation assays. SUMO conjugation assays were carried out in 10 μ l of a reaction mix containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, 0.6 unit/ml inorganic pyrophosphatase, 1 μ g of recombinant SUMO-1, SUMO-2, or SUMO-3, 500 ng of Ubc9, and 100 ng of Sae1/Sae2 and either ³⁵S-radiolabeled c-Fos (4 μ l) or glutathione S-transferase (GST)-c-Fos (500 ng) as previously described (46). Reaction mixes were incubated at 30°C for 2 h and stopped by addition of SDS-containing electrophoresis loading buffer. To assess the effect of PIAS E3s, only 50 ng Ubc9 was used together with 100 ng of recombinant PIAS-1 or of PIAS-x β .

Immunoprecipitation and in vitro pull-down assays. Immunoprecipitation conditions have been described (20). GST-TBP (69), GST-CBP-(451-721) (39), and GST-c-Fos were purified from *Escherichia coli* using the BPERII reagent (Pierce) and bound to glutathione-agarose beads (Sigma). For each pull-down assay, beads (a quantity loaded with 2 μ g of GST fusion proteins) were first saturated with 2 mg/ml bovine serum albumin for 1 h. They were then incubated with 20 μ l of in vitro-translated c-Jun and c-Fos proteins as indicated in Fig. 6A and C in a total volume of 200 μ l of binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% NP-40, 5% glycerol, 2 mM dithiothreitol, protease inhibitors) for 1 h at room temperature. Beads were then washed three

times in the same buffer. Bound proteins were eluted directly in SDS-containing electrophoresis loading buffer.

Quantitative analysis of luminograms and autoradiographs. For quantitative analysis, the relative amounts of proteins were quantified by densitometer scanning of luminograms exposed for various periods of time to take into account both the nonlinear response of Kodak X-OmatAR films and signal differences between sumoylated and nonsumoylated proteins. Tracks with identical intensities were taken as internal references for comparison of the different luminograms. The treatment of the data was carried out using the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image>). All quantitations reflect at least three independent experiments.

Immunofluorescence assays. HeLa cells grown on coverslips were transfected with 3 μ g of each expressing vector using the calcium phosphate coprecipitation procedure; 16 h later, cells were fixed with 4% paraformaldehyde. Indirect immunofluorescence analysis was carried out using the sc52 anti-c-Fos antibody by confocal microscopy.

Cell fractionation experiments. We washed 1.5×10^6 HeLa cells once with phosphate-buffered saline, pelleted them down by centrifugation, resuspended them in 400 μ l of cold buffer A [10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 5 mM dithiothreitol, 1 μ g/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 0.5% Triton X-100], and disrupted them by several passages through a needle; 200 μ l was saved and supplemented with 50 μ l of 5 $\times$ SDS-containing Laemmli sample buffer and used as the total extract. The other 200 μ l of lysed cells were incubated at room temperature for 5 min and then centrifuged at $10,000 \times g$ for 1 min at 4°C; 50 μ l of 5 $\times$ Laemmli sample buffer was added to the supernatants, which constituted the supernatant fraction. The pellets were washed once with buffer A and centrifuged at $10,000 \times g$ for 1 min at 4°C; 50 μ l of 5 $\times$ Laemmli sample buffer was added to the supernatants, which constituted the wash fraction. The pellets corresponding to the nonsoluble fractions were homogenized in 200 μ l of buffer A containing 0.5% Triton X-100 plus 50 μ l of 5 $\times$ Laemmli sample buffer to constitute the pellet fraction. All samples were boiled for 10 min before equivalent volumes were loaded onto electrophoresis gels. An anti-Phax monoclonal antibody (8G5; gift of E. Bertrand) was used to characterize the soluble fraction, and an anti-topoisomerase I antibody (gift of J. Tazi) was used to characterize the insoluble one.

RESULTS

c-Fos is modified by SUMO on lysine 265 in vitro. To determine whether c-Fos can undergo SUMO modification in vitro at lysine 127, 191, or 265, these amino acids were mutated to arginines either individually (c-FosK127R, -K191R, and -K265R) or in combination (c-Fos-K127R,K191R,K265R), and in vitro-translated mutants were compared to c-Fos in an assay containing recombinant Sae1/Sae2, Ubc9, and all three SUMO forms. Proteins harboring an intact K265 (see c-Fos in Fig. 1A; not shown for the others) were SUMO modified, whereas those with a K265R mutation were not (see c-FosK265R in Fig. 1A; not shown for the others). Purified recombinant c-Fos and c-FosK265R proteins were also subjected to the same assay with a similar outcome (Fig. 1B), making it unlikely that, in Fig. 1A, K127 and K191 were masked through nonspecific interactions with proteins of the reticulocyte lysate. Thus, c-Fos is a substrate for sumoylation on K265 in vitro.

c-Fos is modified by SUMO on lysine 265 in vivo. c-Fos is expressed at undetectable levels in most cycling cells. However, mitogen addition to serum-deprived cells, which is one of the most common experimental system for studying the mechanisms of *c-fos* gene regulation (44), induces its rapid and transient expression. For example, in serum-stimulated human HeLa cells, which were largely used below, c-Fos expression peaks 1 h postinduction and returns to basal levels 4 to 6 h later (Fig. 2A). Upon cell lysis in a nondenaturing buffer (not shown), c-Fos appears as a unique smeared band with an apparent molecular mass of 55 to 65 kDa in immunoblotting

FIG. 1. In vitro sumoylation of c-Fos and c-Jun. (A) Sumoylation of in vitro-translated c-Fos and c-FosK265R. Proteins were radioactively translated in the rabbit reticulocyte lysate and subjected to sumoylation in the presence of Sae1/Sae2, Ubc9, and either SUMO-1, SUMO-2, or SUMO-3 (see Materials and Methods). In vitro-translated c-Fos appears as a doublet (bracket) because of internal translation initiation. Sumoylated c-Fos is indicated. (B) Sumoylation of recombinant c-Fos. c-Fos and c-FosK265R were produced in *E. coli* and subjected to sumoylation as for panel A. Detection of unmodified and modified proteins was achieved by immunoblotting using the sc52 anti-c-Fos antibody.

experiments. This molecular heterogeneity reflects differential phosphorylation at multiple sites (see below). In contrast, lysis under denaturing conditions allowed detection of another, slower migrating band of 75 to 85 kDa (Fig. 2A). Importantly, this retarded species is also observed upon serum stimulation of mouse NIH 3T3 cells and BALB/c embryo fibroblasts as well as human HEK293 cells (not shown). The fraction of the retarded species varied between 5 and 12% of total c-Fos depending on the cell type. The shift of 20 kDa, as it has been reported for a number of proteins (31), is consistent with monosumoylation of c-Fos and the lability of the retarded species in a nondenaturing buffer with that of SUMO removal by SUMO hydrolases (31).

To formally establish this, we used HeLa cells stably expressing His₆-tagged versions of SUMO-1, SUMO-2, or SUMO-3, which permits purification of SUMO conjugates by nickel affinity chromatography under denaturing conditions. Immunoblotting analysis of SUMO conjugates showed that serum-induced endogenous c-Fos is modifiable by all three SUMO isoforms in vivo (Fig. 2B). Notably, in contrast to c-Fos sumoylation by SUMO-1, in which only a single sumoylated band was detected, ladders of sumoylated species were observed in the case of SUMO-2 and SUMO-3. Their weaker intensity, however, indicated that they constitute minor species. Whether they correspond to oligo-SUMO-2 and -SUMO-3 chains, formed owing to the presence of a consensus conjugation site (not present on SUMO-1) on SUMO-2 and SUMO-3 (31, 55) and/or to multiple monosumoylations, is not yet clear. This has not been investigated further because of their low abundance and our difficulty in detecting them.

To identify at which site c-Fos is sumoylated in vivo, a mutant (8) in which all 14 lysines were mutated into arginines as well as c-FosK127R, -K191R, -K265R, and -K127R,

FIG. 2. c-Fos modification by SUMO in vivo. (A) Expression kinetics of endogenous c-Fos in serum-stimulated HeLa cells. Cells were stimulated by addition of serum as described in Materials and Methods. They were lysed in a SDS-containing sample buffer at different time points poststimulation and proteins were subjected to immunoblotting analysis with the sc52 anti-c-Fos antibody. bb corresponds to a nonspecific background band detected by sc52 and comigrating with the fastest-migrating c-Fos species. Nonsumoylated c-Fos is indicated by a bracket. (B) c-Fos modification by the three SUMO forms. Asynchronous parental and His₆-SUMO-1-, His₆-SUMO-2-, or His₆-SUMO-3-expressing HeLa cells were stimulated (+) or not (–) by serum for 1 hour to induce endogenous c-Fos expression. Cells were then lysed in a guanidinium HCl-containing buffer and His₆-SUMO conjugates were affinity chromatography purified. Those were analyzed by immunoblotting with the sc52 antibody as well as a fraction (1%) of the total extract (Input) to verify comparable induction of c-Fos in the four cell lines. (C) K265 is the target for SUMO conjugation. Wild-type and K-to-R mutants were transfected in asynchronous HeLa cells together with a His₆-SUMO-2 expression plasmid. SUMO conjugates and a fraction of the total extract were analyzed as for panel B. (D) SUMO conjugation to K265 during the G₀/G₁ phase transition. *c-fos*^{−/−} f10 cells were stably transfected with vectors reproducing the transient expression of the natural *c-fos* gene upon serum stimulation and expressing c-Fos or c-FosK265R. Transient expression can be achieved owing to the presence of a minimal *c-fos* promoter containing the serum-responsive element (pSRE) and of the 3′ untranslated region, which carries the main mRNA instability determinants (3). Cells were arrested in G₀ by serum deprivation for 36 h, stimulated with 20% serum, and treated as for panel A.

K191R,K265R were cotransfected into HeLa cells with either His₆-SUMO-1, His₆-SUMO-2, or His₆-SUMO-3 plasmids. The absence of retarded species in the case of c-FosK/R confirmed that sumoylation actually occurs on a lysine in vivo. Only c-Fos proteins harboring an intact K265 were modified by each of the three SUMOs, indicating that this lysine is the main, if not the unique, sumoylation site in cycling HeLa cells. Typical results are presented for SUMO-2 in Fig. 2C and similar data were obtained in the case of SUMO-1 and SUMO-3 (not shown).

Next, we controlled for the fact that K265 is also the SUMO

acceptor site during the G₀/G₁ phase transition induced by serum stimulation of quiescent cells. c-Fos and c-FosK265R were cloned in a serum-inducible expression vector faithfully reproducing the expression of the natural *c-fos* gene (3), and the plasmids were stably transfected into *c-fos*^{−/−} f10 mouse embryo fibroblasts (10). Transfectants were subsequently arrested in G₀, stimulated with serum, and analyzed for c-Fos protein expression. Figure 2D shows that the K265R mutation did not modify c-Fos expression kinetics but abolished its sumoylation during the G₀ to G₁ transition.

Taken together, these results are consistent with our *in vitro* data and indicate that c-Fos is modified largely, if not only, on a single lysine by SUMO in living cells.

c-Fos sumoylation is a reversible event. We next addressed whether c-Fos sumoylation is a dynamic process regulated by intracellular signaling in different experimental settings. First, the fraction of endogenous c-Fos that is sumoylated was analyzed at various times poststimulation by serum. Figure 3A shows that it reaches a peak at 1 to 2 h and diminishes afterwards. As c-Fos is no longer translated 1.5 h after stimulation, due to the disappearance of its mRNA (44), this was suggestive of either preferential degradation of sumoylated c-Fos over that of the nonmodified protein or progressive desumoylation. Then, we took advantage of the observation that cycloheximide treatment at the peak of induction in serum-stimulated cells leads to both stabilization and increased phosphorylation of c-Fos via a yet-to-be-identified mechanism (51).

The results presented in Fig. 3B show that the amount of sumoylated endogenous c-Fos in serum-stimulated HeLa cells was decreased in the presence of cycloheximide, whereas the bulk of sumoylated proteins, as assayed by immunoblotting with anti-SUMO antibodies, was not affected (not shown). The electrophoretic mobility of sumoylated c-Fos was lower in this experiment, due to cycloheximide-induced phosphorylation. Because protein synthesis inhibition by cycloheximide blocks c-Fos degradation (51), low sumoylation levels are due to desumoylation.

Next, we resorted to heat shock because it induces *c-fos* gene expression and increases AP-1 activity (18). Serum-stimulated HeLa cells were shifted to 43°C at the peak of c-Fos induction, which led to the disappearance of SUMO-modified c-Fos in less than 10 min (Fig. 3C) with no effect on the bulk of sumoylated proteins in the time frame of the experiments (not shown). This was due to desumoylation and not to accelerated degradation of c-Fos because the same effect was observed in the presence of the proteasome inhibitor MG132 (not shown). Moreover, the effect is reversible since, upon return of cells to 37°C, c-Fos sumoylation is restored to the original level within 1 h (not shown). Thus, these experiments suggest that sumoylation of c-Fos is subject to regulation and is a reversible event.

c-Fos sumoylation is not down-regulated by Erk1/2 and Rsk1/2 kinases. c-Fos, which contains approximately 80 serines, threonines, and tyrosines, is phosphorylatable at numerous sites, not all of which are identified. It can be phosphorylated by a number of kinases *in vitro* and by Erk1/2, Rsk1/2, and Erk5 as well as an uncharacterized oncogenic Ha-Ras-activated kinase and possibly others *in vivo* (1, 2, 12, 17, 38, 60, 61). As (i) modifications by Erks and Rsks entail slower electrophoretic mobilities and (ii) low sumoylation levels correlated with accumulation of phosphorylated c-Fos in Fig. 3A and B, we investigated the possible antagonism between sumoylation and phosphorylation by Erk1/2 and Rsk1/2.

Serine 374 and serine 362 are the primary sites targeted by Erk1/2 and the mitogen-activated protein kinase-activated kinases Rsk1/2 (12, 13, 37, 38, 41), respectively. Their phosphorylation leads to protein stabilization (3, 13, 20, 41). Threonine 325 and threonine 331 are secondary targets of Erk1/2; their modification occurs only when serines 362 and 374 are phosphorylated and Erk1/2 activation is sufficiently sustained (37, 38). This enhances the transcriptional activity of c-Fos (34).

FIG. 3. Regulation of c-Fos sumoylation. (A) Fraction of sumoylated c-Fos at various times post-serum stimulation. HeLa cells were serum deprived and stimulated as in Fig. 2A. Proteins samples were prepared at different times poststimulation and subjected to immunoblotting analysis using the sc52 anti-c-Fos antiserum. Luminograms exposed for different periods of time were analyzed by densitometry and the relative amounts of both c-Fos and sumoylated c-Fos were determined using the NIH Image software to plot the relative fractions of sumoylated c-Fos as a function of time; 1 was arbitrarily chosen as the maximal level of sumoylation of c-Fos found at time 1 h after addition of serum. The average of three independent experiments is presented. Similar results were obtained in experiments with fewer time points. (B) Inhibition of c-Fos sumoylation upon protein synthesis inhibition. HeLa cells were stimulated with 20% serum and 50 μ g/ml cycloheximide was added 30 min post-serum addition; 30 min later, cells were lysed for immunoblotting analysis as for Fig. 2A. (C) Desumoylation of c-Fos upon heat shock. HeLa cells were stimulated with 20% serum for 1 hour, at which time they were heat shocked at 43°C for various periods of time. The immunoblotting analysis was conducted as for panel A. bb, background band detected by sc52. c-Fos-P, c-Fos-SUMO, and c-Fos-P-SUMO indicate phosphorylated, sumoylated, and phosphorylated/sumoylated c-Fos, respectively. The cell extracts used in panels B and C were also immunoblotted with anti-SUMO antibodies with no detectable variation in the bulk of sumoylated proteins between the various samples.

Sumoylation of phosphomimetic mutants in which serines 362 and 374 and threonines 325 and 331 were mutated to aspartic acids was therefore analyzed in transient transfection assays and showed no significant decrease in sumoylation (Fig. 4Aa). Moreover, inhibition of the Erk1/2-Rsk1/2 pathway with the pharmacological inhibitor UO126 (16) in a serum stimulation

FIG. 4. Influence of phosphorylation on c-Fos sumoylation. (A) Relative sumoylation levels of various c-Fos phosphorylation site mutants. Exponentially growing HeLa cells were transfected with plas-

experiment did not change endogenous c-Fos sumoylation (Fig. 4B). UO126 did not affect c-Fos desumoylation in cycloheximide-treated or heat-shocked cells either (not shown). Taken together, these experiments argue against a primary role for inhibition of c-Fos sumoylation by Erk1/2 and Rsk1/2-dependent phosphorylation.

Regulation of c-Fos sumoylation by phosphorylation of threonine 232. Threonine 232 is the last of the five phosphoacceptor sites that have been precisely mapped in c-Fos, but how its modification is regulated is poorly understood. Phosphorylation of T232 is not detectable in exponentially growing, c-Fos-expressing cells (17). However, it can be induced upon expression of oncogenic Ha-Ras (Ha-RasG12V) (17). Although only a fraction of c-Fos is modified under these conditions, the biological response may still be elevated, as phosphorylation of T232 entails superactivation of c-Fos transcriptional activity (4, 17). The kinase involved is still unidentified but is neither Erk1/2 nor a Jnk (17). Interestingly, mutation of threonine 232 to aspartic acid (c-FosT232D) decreased SUMO modification by 2- to 2.5-fold upon transfection of HeLa Cells (Fig. 4Aa). In contrast, the nonphosphorylatable mutant c-FosT232A and the wild-type protein were sumoylated to the same extent (Figs. 4Ab), which is consistent with the fact that threonine T232 is at best poorly modified in exponentially growing cells (Fig. 4C).

mids expressing the various mutants under standardized conditions to achieve similar expression levels in the presence of a c-Jun expression plasmid and analyzed by immunoblotting using the sc52 anti-c-Fos antiserum. Relative amounts were determined from densitometer analysis of luminograms. The data are the results of three independent experiments; 1 was arbitrarily chosen as the ratio of sumoylated c-Fos versus nonmodified c-Fos in reference c-Jun plus c-Fos transfections. In these experiments, the fraction of wild-type c-Fos that was sumoylated was approximately 8%. (a) Comparison of the various phosphomimetic mutants. (b) Comparison to that of threonine 232 alanine and aspartic acid mutants. (B) Effect of the inhibition of the Erk mitogen-activated protein kinase pathway on sumoylation of c-Fos. HeLa cells were stimulated by serum as for Fig. 2A. UO126 was added at a concentration of 10 μ g/ml 20 min post-serum stimulation. Cell extracts were prepared at the indicated times for immunoblotting analysis. Immunoblots were probed with anti-Fos, anti-Erk, and anti-phospho-Erk1/2 antisera. The latter permitted visualization of the efficiency of Erk1/2 inactivation. Identical results were obtained when UO126 was added 10 or 30 min after the addition of serum. bb indicates a background band in the case of c-Fos, which is different from that seen in the experiments presented in the other figures due to a difference in antibody batch. (C) Detection of T232-phosphorylated c-Fos in Ha-RasG12V-expressing cells. HeLa cells were cotransfected with either c-Fos and c-Jun expression plasmids (also see Fig. 8) or the same plasmids plus a Ha-RasG12V-expressing construct. Immunoblots of total cell proteins were probed with either the sc52 antibody, to detect the bulk of c-Fos (posttranslationally modified or not), or the anti-phospho-T232 antiserum, which permits visualization of only the fraction of c-Fos modified on T232. Because only a fraction of c-Fos is modified on T232 and to a likely lower titer of the anti-phospho-T232 antiserum compared to the sc52 anti-c-Fos antibody, signal intensities were higher in the latter case than in the former. The luminograms presented are, therefore, not representative of the relative abundances of nonphosphorylated and T232-phosphorylated c-Fos proteins. Short-exposure-time luminograms (S) were selected to visualize the bulk of sumoylated and nonsumoylated c-Fos by the sc52 antiserum, whether Ha-RasG12V is expressed or not. In contrast, long exposure times (L) are presented in the case of detection with the anti-phospho-T232 antiserum to make it clear that no sumoylated c-Fos is detected with the anti-phospho-T232 antiserum.

Next, we asked whether Ha-RasG12V-dependent phosphorylation of T232 could modify c-Fos sumoylation in transfected HeLa cells. Ha-RasG12V led to increased accumulation of c-Fos due to stabilization of the protein via Erk- and Rsk-mediated phosphorylation of S362 and S374 (3, 13, 20, 41). However, the percentage of sumoylated c-Fos was identical to that in control cells not expressing Ha-RasG12V. This was not surprising, as only a limited fraction of c-Fos undergoes phosphorylation on T232 under these experimental conditions. Consistent with this, similar fractions of wild-type c-Fos and of c-FosT232A were sumoylated when these proteins were compared in transfection assays in (i) HeLa cells transfected with Ha-RasG12V, (ii) HCT116 human colon carcinoma cells naturally expressing a mutated Ha-Ras gene, and (iii) mouse embryo fibroblasts transformed by Ha-RasG12V retroviral transfer (not shown). More interestingly, only nonsumoylated c-Fos could be detected in extracts of Ha-RasG12V-transfected HeLa cells probed with an antiserum specific for a phospho-T232 c-Fos peptide (Fig. 4C), even after long exposure of the immunoblots.

Although it is impossible to formally exclude that a SUMO moiety on K265 could reduce access to phosphorylated T232, even after protein denaturation in the presence of SDS and under reducing conditions, these data are consistent with the c-FosT232D mutant analysis presented in Fig. 3A and further support the idea that phosphorylation of T232 can antagonize the sumoylation of c-Fos.

Next, we investigated whether phosphorylation of T232 might explain the variations of c-Fos sumoylation during the G₀/G₁ transition and after either cycloheximide treatment or heat shock of serum-stimulated cells. No induction of T232 phosphorylation was detected in either situation (not shown), indicating that, at most, a very minor fraction of c-Fos, not detectable by immunoblotting, might be modified when Ras is not oncogenically activated. This suggests that there are T232-dependent and T232-independent mechanisms regulating c-Fos sumoylation functioning under different conditions.

Sumoylation of c-Fos/c-Jun down-regulates AP-1 transcriptional activity. We next addressed the role of c-Fos sumoylation. We could exclude that sumoylation constitutes a transient event necessary for protein entry into the nucleus because c-FosK265R accumulates quantitatively in this compartment, as assayed by indirect immunofluorescence (not shown), or for c-Fos degradation, because c-Fos and c-FosK265R accumulated to the same level and showed the same half-lives in pulse-chase experiments (not shown). Therefore, we tested whether sumoylation could control c-Fos transcriptional activity. We investigated this in the context of c-Fos/c-Jun AP-1 heterodimers, whose formation is largely favored over c-Jun/c-Jun homodimers when both proteins are expressed together (14).

Initially, it was necessary to identify the second sumoylation site in c-Jun. Since c-Jun K257 lies in a consensus sequence for sumoylation (IKAE), we tested the sumoylation of single and double K229 and K257 mutants in vitro in the presence of PIAS β . c-Jun turned out to be modified twice by SUMO, c-JunK229R and c-JunK257R were modified once, and c-JunK229R,K257R was not modified at all (Fig. 5). The same results were also obtained with PIAS1 (not shown). This identified K257 as the second c-Jun sumoylation site.

FIG. 5. Sumoylation of c-Jun on K229 and K257. Wild-type and mutant c-Jun in which either K229 or K257 or both were mutated in arginines were subjected to sumoylation in the presence or absence of recombinant PIAS β under the same conditions as for Fig. 1A except that Ubc9 was used at a 10-fold lower concentration. c-Jun appears as multiple bands (bracket) when translated in the reticulocyte lysate. Sumoylated bands are indicated by arrows.

Next, we used transient transfection of HeLa cells to monitor the ability of various variant c-Fos/c-Jun dimers to transactivate a luciferase reporter gene under the control of the human collagenase 1 promoter. This is a well-studied promoter containing a unique TRE/AP-1 site that responds to AP-1 under physiological conditions. Mutation of c-Jun K229 to arginine led to a modest (35%) increase of c-Jun homodimer transactivation activity in HeLa cells (35) that was further increased (80%) by mutation of the second acceptor site (c-Jun K229R,K257R) (Fig. 6A). The observed differences did not reflect different protein steady-state levels, as verified by immunoblotting (not shown).

The transactivation properties of c-Fos/c-Jun heterodimers bearing different numbers of sumoylatable lysines were studied under the same normalized conditions of transfection (Fig. 6A). These data can be summarized as follows: (i) the highest transcription activity was observed with dimers containing no sumoylation sites (fourfold higher than that of wild-type c-Fos/c-Jun dimers), (ii) the presence of a single sumoylation site, whatever its position in c-Fos or c-Jun, is sufficient to reduce transcription activity relative to c-FosK265R/c-JunK229R, K257R dimers, and (iii) c-Fos/c-Jun dimers with two available sumoylation sites show activity intermediate between that of wild-type dimers and dimers with a single sumoylation site. It must, however, be stressed that the differences observed in these experiments probably underestimate the potency of inhibition by sumoylation because only small amounts ($\leq 5\%$) of both wild-type c-Fos and c-Jun were sumoylated in our transfection experiments, as assayed by immunoblotting (not shown).

As a next step, we wanted to establish that increased transactivation activity of c-FosK265R was due to the inability of K265 to be sumoylated and not to modification of other lysines possibly induced by conjugation of SUMO to K265. We compared the activity of the c-FosK/R mutant, in which all lysines were turned into arginines, with that of a mutant in which K265 was the only nonmutated lysine (c-FosK/R,K265). As expected, c-FosK/R,K265 was a less efficient transactivator than

FIG. 6. Inhibition of c-Fos and c-Jun sumoylation stimulates AP-1-dependent transcription activity. (A) Stimulation of AP-1 activity by non sumoylatable c-Fos and c-Jun. We transfected 0.4×10^6 asynchronous HeLa cells with (i) 200 ng of a luciferase reporter plasmid under the control of the collagenase promoter, (ii) 500 ng of an EGFP expression vector used for normalization, and (iii) 50 ng of expression plasmids for wild-type or mutant c-Fos and c-Jun as indicated. The different motifs of the bars indicate the number of SUMO acceptor sites present within c-Fos/c-Jun AP-1 complexes. In the initial calculation, fold activation values were calculated as the ratio between luciferase activity and EGFP fluorescence. The activity of the wild-type c-Fos/c-Jun dimer was used as an internal reference and arbitrarily set to 1. The results shown are the means of four independent experiments $\pm$ standard deviation. (B) Sumoylation of c-FosK/R and c-FosK/R,K265. HeLa cells were transfected with the indicated constructs together with a His₆-SUMO-2 expression plasmid and SUMO conju-

c-FosK/R in the presence of either c-Jun or c-JunK229R, K257R (Fig. 6C). Notably, c-FosK/R,K265 is sumoylated to the same extent as wild-type c-Fos (Fig. 6B), confirming that lysine 265 is the major, if not the unique, sumoylation site in c-Fos.

In conclusion, our data are consistent with the idea that sumoylation, either on K265 of c-Fos or on K229 or K257 of c-Jun, entails lower c-Fos/c-Jun AP-1 heterodimer activity.

Transcriptional activity of c-Fos/SUMO chimeric proteins. Mutation of lysines to arginines may have indirect effects on transcriptional activity, in particular because lysines are also sites for numerous posttranslational modifications. We therefore wished to further support our hypothesis that transcriptional activity of c-Fos is reduced by sumoylation. To this aim, we tested whether a modification mimicking sumoylation at the level of K265 would attenuate AP-1 transactivation. Due to the multiplicity of transactivation domains in c-Fos (9, 28, 59), the removal of the domain lying downstream of K265, while reducing the intrinsic transcriptional activity under the experimental conditions used by fivefold, does not abolish it.

We exploited this favorable situation to study a c-Fos/SUMO chimera in which SUMO-1 and SUMO-2 were fused to c-Fos truncated at position 265. The constructs [c-Fos(1-265)-SU1 and c-Fos(1-265)-SU2, respectively; Fig. 7A] included a flexible (Ser-Gly)₃ linker expected to allow the SUMO moiety to position in a spatial configuration resembling the natural one. To avoid conjugation of c-Fos-SUMO chimeras to other cell proteins, the C-terminal diglycine motif of each SUMO, which is required for isopeptide bond formation, was mutated to a dialanine (43). As control proteins, we also constructed two other chimeras: c-Fos(1-265)-EGFP, in which the fluorescent enhanced green fluorescent protein (EGFP) was grafted on c-Fos at position 265 using the (Ser-Gly)₃ linker; and SU1-c-Fos(1-265), in which SUMO-1 was N-terminally fused to c-Fos(1-265) (Fig. 7A). Indirect immunofluorescence assays showed that all chimeras accumulated constitutively within the nucleus with the characteristic diffuse, granular, and extranucleolar pattern of c-Fos (not shown). Of note, the c-Fos(1-265) and c-Fos/SUMO chimeras accumulated to comparable levels, indicating that addition of SUMO neither accelerates nor decreases c-Fos(1-265) turnover.

The transactivation activity of truncated c-Fos(1-265) was compared to those of c-Fos(1-265)-EGFP and the various c-Fos(1-265)-SUMO chimeras in an assay involving c-JunK229R,K257R. This c-Jun mutant was chosen to avoid any interference between SUMO moieties on the two components of the c-Fos/c-Jun heterodimers. C-terminal fusion of SUMO-1 and SUMO-2 to the truncated c-Fos led to a 10-fold decrease in AP-1 transcriptional activity whereas both C-terminal grafting of EGFP and N-terminal addition of SUMO only diminished transcription twofold (Fig. 7B). C-terminal

gates were purified and analyzed by immunoblotting with the sc52 anti c-Fos antibody as in Fig. 2B. (C) Transactivation properties of c-FosK/R and c-FosK/R,K265. This experiment was performed as for panel A with the indicated plasmids. The activity of the c-FosK/R,K265/c-Jun dimer, which is identical to that of the c-Fos/c-Jun dimer in panel A, was taken as the internal reference and arbitrarily set to 1. The results presented are the means of at least three independent experiments.

FIG. 7. Inhibition of AP-1-dependent transactivation by chimeric c-Fos/SUMO proteins. (A) Structure of c-Fos(1–265)–SUMO chimeras. The C-terminal diglycine motifs of SUMO proteins was mutated into dialanines. In c-Fos(1–265)–SU-1 and –SU-2 as well as in c-Fos(1–265)–EGFP, the SUMO and EGFP moieties were separated from c-Fos(1–265) by a flexible (Ser-Gly)₃ linker. In the case of Su1-c-Fos, no linker was used between SUMO-1 and c-Fos. (B) Transactivation assays. We cotransfected 0.4×10^6 asynchronous HeLa cells with 200 ng of a luciferase reporter plasmid under the control of the collagenase promoter, 50 ng of the c-JunK229R,K257R, and appropriate concentrations of the other plasmids in order to express similar protein levels; 200 ng was used for the reference c-Fos(1–265) expression plasmid, and optimal concentrations for the other vectors were determined experimentally in preliminary transfection experiments in which Fos chimera protein levels were assayed by immunoblotting using an anti-c-Fos antiserum. Luciferase activity was measured 24 h posttransfection. Fold activation values correspond to the ratio between luciferase activity and EGFP fluorescence. The reference value obtained with the c-Fos(1–265)/c-JunK229/257R plasmids was arbitrarily set to 1. The results shown are the means of at least three independent experiments $\pm$ standard deviation. The intrinsic transactivation activity of c-Fos(1–265) was fivefold less than that of wild-type c-Fos in control experiments. (C) Structure of c-Fos–SUMO1 chimeras. Constructs were made as in panel A except that full-length c-Fos was used instead of c-Fos(1–265). (D) Transactivation assays. Experiments were conducted as for panel B.

grafting of SUMO-3 to c-Fos(1–265) resulted in an effect comparable to that of SUMO-1 and SUMO-2 in separate experiments (not shown).

To complement this, we fused SUMO-1 to full-length c-Fos. C-terminal but not N-terminal (Fig. 7C) fusion led to a dramatic stabilization, most probably due to the masking of the C-terminal destabilizer of c-Fos operating in exponentially growing cells (20). Therefore, the quantities of expression vectors had to be optimized to allow similar protein accumulation prior to comparison of transactivation activity in transfection assays. The data presented in Fig. 7D show that N-terminal fusion of SUMO led to no change in transactivation activity, whereas C-terminal fusion decreased transcriptional activity 10-fold.

While data obtained with artificial fusion proteins must be interpreted with caution, the fact that the c-Fos(1–265)–SUMO proteins showed an effect opposite that of the non-sumoylatable c-FosK265R mutant argues that sumoylation in the C-terminal moiety of the protein reduces c-Fos transacti-

vation. In contrast, SUMO modification of the N terminus seems to have, at best, a modest effect on transactivation.

Preferential sumoylation of c-Fos in AP-1 dimers. As heterodimerization and binding to DNA are necessary conditions for transactivation by c-Fos, we investigated whether they could either affect or be affected by sumoylation. Two lines of evidence already argued against the inability of sumoylated c-Fos to heterodimerize: (i) c-FosK265R/c-JunK229R,K257R showed lower transactivation activity upon coexpression of c-Fos(1–265)–SU-1 and c-Fos(1–265)–SU-2, most probably because of formation of competing c-Fos(1–265)–SUMO/c-Jun complexes (not shown); and (ii) both sumoylated and non-sumoylated c-Fos and c-Jun bound quantitatively to their dimerization partner *in vitro* (Fig. 8A). We also noted that cotransfection of c-Jun dramatically increased the sumoylation of a transfected c-Fos in asynchronously growing HeLa cells (Fig. 8B, see lanes c-Fos and c-Fos + c-Jun), suggesting that heterodimerization positively influences sumoylation of c-Fos.

FIG. 8. Heterodimerization of sumoylated c-Fos, binding to DNA, and interaction with CBP and TBP. (A) In vitro dimerization with c-Jun. In vitro-translated c-Fos and c-Jun were sumoylated using a Sae1/Sae2-, Ubc9-, and SUMO-1-containing reaction mix. Only a fraction of each protein was sumoylated, which allowed direct comparison of modified and unmodified proteins. A recombinant c-Jun protein was added to the c-Fos reaction (Aa), whereas a GST-c-Fos protein was added to the c-Jun one (Ab). In the former case, complexes were immunoprecipitated using the sc45 anti-c-Jun antibody, whereas in the latter, they were purified by affinity chromatography on glutathione beads. Bound proteins were then resolved by SDS-PAGE. (B) In vivo sumoylation of leucine zipper and DNA binding mutants. HeLa-SUMO-2 cells were transfected with equivalent amounts of plasmids coding for the indicated proteins as described in Materials and Methods. c-Jun proteins were tagged with a Flag epitope. Cell extracts were prepared and subjected to either direct immunoblotting analysis to quantify the fraction of sumoylated c-Fos (Ba and Bb) or immunoprecipitation with an anti-Flag antibody to assay c-Fos and c-Jun mutant dimerization by immunoblotting (Bc). (Ba) Long- and short-exposure luminograms of an immunoblotting analysis of total cell extracts with the sc52 anti-cFos antiserum. (Bb) Fraction of sumoylated c-Fos determined as described for Fig. 3A. The values are the averages of two independent experiments. Relative sumoylation levels were calculated compared to the fraction of sumoylated c-Fos (8% ± 0.3) in the c-Fos plus c-Jun transfection. (Bc) Analysis of c-Fos and c-Jun mutant dimerization. Immunoprecipitations were carried out with the anti-Flag antiserum to pull down c-Jun-containing AP-1 complexes and three types of fractions, corresponding to the same initial volume of extract, were subjected to immunoblotting analysis using the anti-Flag and sc52 antisera to detect c-Jun and c-Fos proteins, respectively, in total extracts (T), immunoprecipitates (IP), and supernatants (S). Similar results were obtained with HeLa-SUMO-1 and HeLa-SUMO-3 cells (not shown). (C) Binding to DNA. The various proteins were produced by in vitro translation (right panel) and tested in electrophoretic mobility shift assays (left panel) using a synthetic ³²P-labeled TRE probe. Competition was done with increasing quantities of an unlabeled TRE or a scrambled probe. The c-Jun/c-Jun homodimer, which binds less efficiently to DNA than c-Fos/c-Jun homodimers, is not visible in the figure.

This was confirmed by the analysis of a dimerization-deficient mutant lacking the leucine zipper (c-Fos Δ LZ) and a mutant with decreased dimerization efficiency (c-FosVAV), both of which showed less sumoylation than the wild-type protein in the presence of exogenous c-Jun (Fig. 8B).

Next, we asked whether binding to DNA was necessary for sumoylation of c-Fos. We first assayed sumoylation of DNA-binding-deficient c-Fos mutants. Neither deletion of the DNA-binding domain (c-Fos Δ DBD) nor point mutations within it (c-FosVV) entailed decreased sumoylation (Fig. 8B), suggesting that binding to DNA is not an absolute prerequisite for sumoylation. This conclusion was confirmed in two other experiments: (i) cotransfection of a c-Jun mutant (c-Jun Δ ARK), capable of dimerization with c-Fos but unable to bind to AP-1 motifs, did not alter the degree of c-Fos sumoylation; and (ii) a c-Fos variant (c-Fos-A), in which the DNA-binding domain was replaced by an acidic domain that enhances the stability of interaction with c-Jun but abolishes binding to DNA (42), showed sumoylation comparable to that of wild-type c-Fos in the presence of c-Jun (Fig. 8B). Finally, we tested whether sumoylation of c-Fos could prevent or destabilize the binding of c-Fos/c-Jun dimers to DNA in an electrophoretic mobility shift assay. Heterodimers composed of c-Jun and either c-Fos, c-Fos(1-265), or c-Fos(1-265)-SU-1 bound equally well to the TRE probe, as shown in competition experiments with an excess of unlabeled probe (Fig. 8C).

Thus, our data indicate that c-Fos is preferentially sumoylated when heterodimerized with c-Jun and suggest that inhibition of dimerization is not the primary reason for lower transactivation activity of c-Fos. They also show that, even though binding to DNA is not an absolute prerequisite for modification of c-Fos by SUMO, sumoylated c-Fos can bind to AP-1 motifs *in vitro* with an affinity comparable to that of the nonsumoylated protein.

Sumoylation of c-Fos does not inhibit the interaction of c-Fos/c-Jun dimers with CBP and TBP *in vitro*. As transactivation by AP-1 implies interaction with TBP and CBP, we asked whether the lower transactivation efficiency of dimers containing c-Fos(1-265)-SU-1, c-Fos(1-265)-SU-2, and c-Fos(1-265)-SU-3 (Fig. 7) could be explained by a decreased binding to TBP and CBP. The lower activity could not be accounted for by less efficient binding to c-Fos itself because the motifs responsible for binding to these two cofactors are located between amino acids 340 and 350 (33) and 280 and 380 (5) of c-Fos, respectively, and are absent from all four truncated or chimeric proteins.

As one SUMO on a c-Fos/c-Jun dimer is sufficient to decrease transactivation, we considered the possibility that sumoylation on c-Fos may have an effect *in trans* by affecting the ability of TBP and CBP to interact with the c-Fos/c-Jun heterodimer at the level of c-Jun. As a first step to test this possibility, *in vitro*-synthesized radioactive c-Jun was used to form either homodimers or heterodimers with unlabeled c-Fos-1 to 265 or c-Fos(1-265)-Su1. AP-1 complexes were then tested for their ability to bind to either GST-TBP or a GST fusion made with a truncated CBP protein (CBP451-721) bearing the KIX motif necessary for binding to c-Jun (6). No difference was observed between c-Jun/c-Fos(1-265) and c-Jun/c-Fos(1-265)-SU-1 dimers (Fig. 9A), which is consistent

with the idea that lower transactivation cannot be explained by simple inability of AP-1 heterodimers containing a sumoylated c-Fos to interact with CBP or TBP.

To confirm this, radioactive *in vitro*-translated c-Fos was (i) sumoylated using a sumoylation cocktail, (ii) mixed with unlabeled *in vitro*-translated c-Jun to form heterodimers, and (iii) subjected to pull down by GST-TBP. The data presented in Fig. 9B indicate that AP-1 heterodimers containing sumoylated c-Fos associate with TBP as efficiently as those containing the nonsumoylated protein. Interestingly, in a reciprocal experiment in which radioactive c-Jun was sumoylated, allowed to heterodimerize with c-Fos, and subjected to pull-down experiments with GST-TBP and GST-CBP451-721, AP-1 heterodimers containing sumoylated c-Jun were as efficient as those containing nonmodified c-Jun in interacting with TBP and CBP (Fig. 9C). Thus, decreased activity of AP-1 by sumoylation of c-Fos cannot be explained by an intrinsic inability of sumoylated c-Fos to dimerize with c-Jun or by the inability of c-Fos/c-Jun dimers to interact with CBP and TBP. Additionally, sumoylation of c-Jun does not make c-Fos/c-Jun heterodimers unable to bind to CBP and TBP, at least on its own.

Differential intranuclear distribution of sumoylated and nonsumoylated c-Fos. We next wondered whether sumoylation could affect c-Fos intranuclear distribution. Confocal analysis did not reveal any difference between the localization of c-Fos, c-FosK265R, c-Fos(1-265), and c-Fos(1-265)-SU-1, which all showed a granular intranuclear distribution with nucleolar exclusion (Fig. 10A). Several sumoylated proteins, such as SP100 (58), Lef1 (49), and Tcf4 (67), are found associated with promyelocytic leukemia nuclear bodies. However, cotransfection experiments showed no significant colocalization between promyelocytic leukemia bodies and c-Fos, c-FosK265R, c-Fos(1-265), or c-Fos(1-265)-SU-1 (data not shown).

Certain sumoylated proteins have been found to be enriched in insoluble nuclear matrix-associated structures, such as Polycomb group bodies (29), different from promyelocytic leukemia bodies. We therefore tested whether sumoylated c-Fos was preferentially associated with the insoluble nuclear fraction in serum-stimulated HeLa cells, using fractionation experiments essentially as described by Kagey et al. (29). Phax I, a factor involved in small nuclear RNA export from the nucleus (40), was used to characterize the nucleoplasmic fraction, whereas topoisomerase I, which is essentially associated with chromatin and nucleoli in growing cells (15), was used to characterize the insoluble one. While the nonsumoylated endogenous c-Fos was found equally distributed between the soluble and insoluble nuclear chromatin- and matrix-containing fraction, SUMO-modified c-Fos was entirely associated with the latter fraction (Fig. 10Ba and 10Bb). Similar experiments were carried out with HeLa cells ectopically expressing c-Fos, c-Fos(1-265), and c-Fos(1-265)-SU-1. The ratio of protein associated with the insoluble fraction to that not associated was higher in the case of c-Fos(1-265)-SU-1 than in that of c-Fos(1-265), strengthening the conclusion that sumoylated c-Fos specifically associates with an insoluble nuclear fraction.

DISCUSSION

We report here that c-Fos is sumoylatable by the three SUMO isoforms on lysine 265 and that c-Jun is modifiable by

FIG. 9. Binding of c-Fos-containing AP-1 dimer to TBP and CBP. (A) Binding of AP-1 dimers containing c-Fos-SUMO chimeras to TBP and CBP. c-Jun was produced by *in vitro* translation in a radioactive form and the c-Fos proteins in a nonradioactive one. Heterodimers were formed in the presence of a twofold excess of c-Fos proteins. The homo- and heterodimers were pulled down with GST-TBP or GST-CBP451-721 using glutathione beads and analyzed by SDS-PAGE. c-Jun homodimers appear twice as intense as c-Fos/c-Jun heterodimers because they contained two radioactive molecule instead of one in the case of c-Fos/c-Jun heterodimers. (B) Binding of AP-1 dimers containing a sumoylated c-Fos to TBP. c-Fos was produced by *in vitro* translation in a radioactive form and was *in vitro* sumoylated as in Fig. 1A. It was then mixed with c-Jun translated *in vitro* in a nonradioactive form. The heterodimers were then pulled down with GST and GST-TBP as in panel A and analyzed by SDS-PAGE. The bound proteins were visualized by Phosphorimager analysis. (C) Binding of AP-1 dimers containing a sumoylated c-Jun to TBP and CBP. c-Jun was produced by *in vitro* translation in a radioactive form and was *in vitro* sumoylated. After mixing with c-Fos translated *in vitro* in a nonradioactive form, the heterodimers were then pulled down with GST, GST-TBP, and GST-CBP451-721 and analyzed by electrophoresis.

SUMO on K229 in addition to K257. Transactivation assays involving both c-Fos and c-Jun sumoylation site mutants and chimeric c-Fos-SUMO chimeras strongly suggest that sumoylation at any of the three acceptor sites of c-Fos/c-Jun heterodimers reduces AP-1 transcriptional activity. Reduced transactivation by sumoylated c-Fos is not due to inhibition of protein entry into the nucleus, inability to dimerize or to bind to DNA, or inability of c-Fos/c-Jun dimers to interact with CBP or TBP. However, it correlates with specific association of sumoylated c-Fos with an insoluble nuclear fraction.

Under our experimental conditions, transcription repression cannot be explained by a modification in c-Fos turnover since, on one hand, the nonsumoylatable c-FosK265R mutant displays the same half-life as wild-type c-Fos and, on the other hand, the sumoylation-mimicking constructs c-Fos(1-265)-SU-1, c-Fos(1-265)-SU-2, and c-Fos(1-265)-SU-3 accumulate to the same level as the stabilized C-terminally truncated c-Fos(1-265) mutant, although they are much less efficient in transcription assays. However, if our work excludes that sumoylation is a prerequisite preceding c-Fos destruction, it does not rule out that, upon sumoylation, the natural c-Fos may have its turnover altered: because the C terminus of c-Fos, which is deleted in the c-Fos(1-265)-SU-1, c-Fos(1-265)-SU-2, and c-Fos(1-265)-SU-3 constructs, contains both positive and negative elements controlling protein turnover (3, 8, 20, 41), it is possible that conjugation of SUMO influences their activity in the context of the full-length molecule.

Regulation of c-Fos sumoylation. Variations in the sumoylation level of c-Fos were observed under different conditions, including the G₀/G₁ transition and stress by cycloheximide or heat shock. This suggests that c-Fos sumoylation is both reversible and regulated by intracellular signaling. The analysis of phosphorylation site mutants of c-Fos, together with the lack of effect of the inhibitor UO126, excluded a role for Erk1/2 and Rsk1/2 kinases in this process. Similarly, the use of other kinase inhibitors revealed no role for the phosphatidylinositol 3-kinase/Akt, Jnk, and P38/SAPK pathways in the reduction of c-Fos sumoylation (not shown). However, our experiments suggested an antagonistic role for phosphorylation of threonine 232, which is targeted by an unknown kinase upon expression of an oncogenically activated Ha-Ras (17). As we failed to detect any phosphorylation of T232 in serum-stimulated cells, even when treated by cycloheximide or heat shock, this suggests that reduced sumoylation of c-Fos may be controlled via both T232-dependent and -independent mechanisms operating under different conditions. This will require future investigation.

What is the mechanism(s) for reduced transactivation activity by c-Fos/c-Jun heterodimers? The steady-state level of sumoylated c-Fos is relatively modest (a small percentage of total protein). This is, however, consistent with the levels for other sumoylated transcription factors (22, 24, 25, 27, 36) and suggests that sumoylation preferentially targets a biologically significant fraction of c-Fos capable of preventing transcription by other nonsumoylated molecules. How sumoylation molecularly affects the activity of transcription factors is still poorly understood (22, 25) for various reasons. Particularly, in addition to concerning only a small fraction of the transcription factor, sumoylation is a highly labile modification and no cell-penetrating drug inhibiting desumoylation has been described

FIG. 10. Intranuclear localization of sumoylated c-Fos. (A) Localization of the different constructs. HeLa cells were transiently transfected with plasmids encoding either c-Fos, c-Fos(1–265), or c-Fos(1–265)–SU-1. Cells were fixed 16 h after transfection for immunofluorescence analysis with the sc52 c-Fos antibody. (B) Nuclear fractionation experiments. Soluble and insoluble fractions were prepared as described in Materials and Methods and equal volumes of proteins were analyzed by immunoblotting using the sc52 anti-c-Fos antiserum. (a) HeLa cells were induced for 1 hour with 20% fresh serum. (b) Asynchronous HeLa cells were cotransfected with expression plasmids coding for c-Fos and c-Jun and cell fractionation was carried out 16 h later. (c and d) The same experiments as in b with c-Fos(1–265) and c-Fos(1–265)–SU-1 expression plasmids, respectively. Immunoblots were probed with anti-Phax and anti-topoisomerase I antisera to verify the quality of the fractionations.

so far. Moreover, no antibody strictly specific for given sumoylated proteins has been developed yet, and, finally, the fraction of the protein that is biologically active *in vivo* is difficult to determine and therefore unknown.

As lysines are subject to several types of posttranslational modifications, an important question is whether sumoylation of c-Fos K265 reduces transcription directly or indirectly via inhibition of another transactivation-altering modifications (25). To address this issue, we constructed c-Fos chimeras in which SUMO-1, SUMO-2, and SUMO-3 were grafted at the level of K265 to a truncated c-Fos protein using a flexible linker expected to permit a positioning resembling that of a naturally conjugated SUMO. The data obtained must be interpreted with caution, due to both the artificial nature of these proteins and the lack of the C-terminal domain of c-Fos. However, they do not support the second possibility, as the c-Fos(1–265)–SU-1, c-Fos(1–265)–SU-2, and c-Fos(1–265)–SU-3 chimeras would be expected to show transcriptional activities comparable to those of c-Fos(1–265) or c-Fos(1–265)–EGFP in this case. It is, however, important to stress that these experiments do not exclude that modification of K265 by, for example, ubiquitin or acetyl groups may increase c-Fos transcription activity compared to the unmodified protein.

A second question relates to the role of the site of sumoylation for reducing c-Fos/c-Jun heterodimer activity. Showing a certain degree of flexibility, our c-Fos and c-Jun mutant analysis suggests that one SUMO at any of the three sumoylation sites is sufficient to exert a repressive effect. However, mutation of two or three acceptor lysines into arginines correlates with

slightly higher AP-1 transactivation relative to heterodimers with only one lysine mutation. Further experiments using antibodies specific for sumoylated c-Fos and sumoylated c-Jun will be required to determine whether the highest repression level by SUMO is due to cooperation between different SUMO moieties conjugated to the same c-Fos/c-Jun heterodimer. On the other hand, there appear to be some positional constraints for reduction of c-Fos activity by SUMO. Grafting it to the c-Fos N terminus has no effect on AP-1-dependent transactivation, in contrast to its grafting to the C terminus or at the level of K265. Thus, c-Fos departs from the Sp3 transcription factor, in which N-terminal fusion of SUMO has the same inhibitory effect as natural SUMO conjugation within the C-terminal part of the molecule (47).

One possible explanation is that SUMO masks one or several functional domains located in the vicinity of K265. The fact that EGFP, which displays a globular structure, represses c-Fos transcription activity to some extent when grafted at the level of K265 partially supports this view. However, the much less potent inhibitory effect of EGFP also suggests that elements specific to SUMO are important for efficient repression. Whether this is due to specific interactions of yet unidentified proteins with surfaces contributed by both c-Fos and SUMO will be the topic of subsequent investigations.

c-Fos contains multiple transactivation domains, raising the question of which is affected by sumoylation. T232 resides within one moiety (HOB1 motif; amino acids 226 to 236) of a bipartite transactivation domain of c-Fos, while the LK₂₆₅AE sumoylation site overlaps the N terminus of the other moiety

(HOB2 motif; amino acids 267 to 276). In addition, Ha-Ras-induced phosphorylation of T232 leads to transcriptional superactivation of c-Fos via the cooperation between HOB1 and HOB2 (4, 17, 59). As (i) no sumoylated c-Fos was detected with the anti-phospho-T232 peptide antibody and (ii) the phosphorylation-mimicking c-FosT232D mutant showed decreased sumoylation, it is tempting to propose a functional antagonism between phosphorylation of T232 and sumoylation of K265 in the control of HOB1 and HOB2 activity. Confirmation of this hypothesis as well as the elucidation of the interplay between these two posttranslational modifications will require identification of both the kinase and the SUMO E3 involved. Whether HOB1 and HOB2 activity is reduced by sumoylation or not, the fact that c-Fos(1–265)–SUMO chimeras are less active than c-Fos(1–265) shows that sumoylation can impair the activity of transactivating elements located upstream of HOB1. Moreover, elements that positively influence transcription lie downstream of K265, as indicated by the lower activity of c-Fos(1–265) compared to the full-length protein (see Results). At this stage of investigation, we cannot exclude that sumoylation may affect them as well.

How sumoylation of only a fraction of c-Fos can prevent transcription by a majority of nonsumoylated c-Fos molecules is unclear (25). It has been proposed that SUMO exerts its function by a cyclic process of modification and demodification rather than acting stoichiometrically and that unmodified proteins with a history of sumoylation may also have properties different from those that have never been modified (25, 27). In this model, the transient attachment of SUMO would be sufficient to promote specific protein interactions, induce a conformational change, or trigger subsequent modification to irreversibly alter the function of the substrate despite subsequent demodification.

Sumoylation of c-Fos *in vivo* preferentially occurs in the context of c-Fos/c-Jun dimers and does not seem to affect the ability to dimerize with c-Jun, at least as assayed *in vitro*, which is reminiscent of other transcription factors, such as Sp3 (52), Tcf4 (67), and LEF-1 (49). Moreover, binding to AP-1 sites is not affected by sumoylation of c-Fos in gel shift assays and sumoylation did not block binding to TBP or CBP to c-Fos/c-Jun dimers *in vitro*. It is, therefore, conceivable that sumoylation of c-Fos can occur at the level of target gene promoters. This view is supported by preliminary *in vivo* competition experiments in which overexpression of poorly transactivating c-Fos(1–265)–SU-1 reduced transactivation by the c-FosK265R mutant (not shown). This argues against a scenario in which sumoylated c-Fos would be generated at more or less defined places in the nucleus and then migrate to target promoters to shut off transcription.

Confirmation of this model and determining whether sumoylation reduces transcription coactivator activity and/or promotes recruitment of transcription repressors will require a better understanding of the molecular basis of c-Fos action at the level of its target promoters and the availability of antibodies specific for sumoylated c-Fos to conduct chromatin immunoprecipitation analysis. In this respect, the case of the transcription factor Elk-1 is interesting, since its sumoylation has recently been reported to stimulate the recruitment of histone deacetylase 2 at the level of target promoters (68). However, SUMO conjugation has also been implicated in the

control of its nucleocytoplasmic shuttling, suggesting multiple roles for the same posttranslational modification (50).

Cell fractionation experiments indicated that both sumoylated endogenous c-Fos and the c-Fos(1–265)–SU-1 chimera preferentially associate with an insoluble fraction of the nucleus. An important question is whether sumoylation concerns a fraction of c-Fos protein already localized in this fraction or whether sumoylation induces relocalization of c-Fos into a weakly active chromatin domain, possibly still bound to target promoters, after having reduced its transactivation activity. Along this line, it will be interesting to investigate whether PIAS proteins can promote the intranuclear redistribution because, in addition to stimulating the sumoylation of c-Fos, at least *in vitro* (not shown; also see below), and c-Jun, (i) they are able to interact with SUMO, (ii) PIASy binds specifically to matrix attachment regions (49), and (iii) PIASy can target Lef1 (49) and Tcf4 (67) to promyelocytic leukemia nuclear bodies. Preliminary immunolocalization studies of the c-Fos–SUMO chimeric proteins used in this study did not reveal any colocalization with promyelocytic leukemia bodies, in which a number of SUMO-conjugated substrates have been reported to accumulate (55, 56). It has, however, recently been proposed that other nuclear matrix-associated compartments, such as polycomb bodies (29), may also recruit sumoylated proteins. Whether sumoylated c-Fos is targeted to such structures is currently under investigation.

Multiple effects of sumoylation events in the AP-1 pathway.

It is becoming increasingly clear that sumoylation controls the activity of different proteins involved in the AP-1 pathway in a complex manner, sometimes with opposite actions, which renders investigations particularly difficult. As a matter of fact, PIAS E3-stimulated sumoylation of upstream effectors of c-Jun, such as axin (48) (which activates JNK) and JNK (32), can activate c-Jun-containing AP-1 complexes, whereas direct sumoylation of c-Jun (35) or c-Fos (this work) is inhibitory. Adding to this complexity, sumoylation controls the activity of AP-1 transcriptional coactivators such as CBP/p300 (23) and SRC1 (11) and the Su1Pr SUMO isopeptidase indirectly enhances AP-1 transcription, probably by affecting coactivator localization (7).

The diverse effects of the SUMO pathway on the various components of a specific signaling pathway, as described above in the case of AP-1, may explain the often-made observation that overexpression of SUMO, Ubc9, or PIAS proteins affects the activity of wild-type and nonsumoylatable proteins in the same way (49, 53, 67). Along the same line, overexpression of Ubc9 and a *trans*-dominant negative mutant of Ubc9 in which the active-site cysteine is mutated in a serine led to a similar 30% increase in c-Fos transcription activity (not shown). Additionally, overexpression of PIASx β and PIAS1, despite their ability to stimulate the sumoylation of c-Fos *in vitro* (not shown), led to no modification in c-Fos sumoylation levels. As these E3s may not be limiting in cells, these experiments permitted us neither to confirm nor to invalidate *in vivo* the observation that sumoylation of c-Fos is stimulated by these two E3s *in vitro*.

Further work will be required to fully understand the multiple effects of the SUMO/PIAS system in the AP-1 pathway as well as to determine whether other SUMO E3s are involved in the sumoylation of c-Fos.

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