SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein

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SUMO-1 is a small ubiquitin-related modifier protein that is covalently linked to many cellular and viral protein targets. Modification by SUMO-1 is proposed to play a role in protein targeting and/or stability. We show here that adenovirus type 5 early region 1B 55-kDa (E1B-55kDa) oncoprotein can be covalently modified by SUMO-1 *in vivo* through a major attachment site comprising a single lysine residue at amino acid position 104. The sequence surrounding this lysine matches the proposed ΨKxE consensus motif required for SUMO-1 conjugation. A single mutation (K104R) that abolishes SUMOylation of E1B-55kDa dramatically reduces the ability of the adenovirus type 5 protein to transform primary baby rat kidney cells in cooperation with E1A and to inhibit p53-mediated transactivation. Overexpression of SUMO-1 in adenovirus type 5 E1A/E1B-55kDa-transformed baby rat kidney cells causes the relocalization of E1B-55kDa from the cytoplasm to the nucleus, where it accumulates with SUMO-1 in dot- or track-like structures. Significantly, when SUMO-1 is ectopically expressed in transformed rat cells no effect on the cytoplasmic localization of the E1B-K104R mutant protein is observed. Our results demonstrate that SUMO-1 modification is required for transformation by adenovirus type 5 E1B-55kDa and provide further evidence for the idea that this posttranslational modification plays a role in protein targeting to specific subcellular sites.

The 55-kDa phosphoprotein encoded in early region 1B (E1B-55kDa) from adenovirus type 5 (Ad5) is required for efficient viral DNA replication, selective viral late mRNA transport to the cytoplasm, and shut-off of host cell protein synthesis in productively infected cells (reviewed in ref. 1). In addition, the Ad protein provides functions for complete oncogenic transformation of mammalian cells in cooperation with Ad E1A (2). During the past few years it has been well established that the transforming potential of E1B-55kDa correlates with its ability to act as a direct transcriptional repressor targeted to p53-responsive promoters by binding to the tumor suppressor protein (3, 4). Considerable evidence suggests that these activities antagonize p53-induced apoptosis (5) and/or cell cycle arrest (6). The regions required for transformation map to several segments in the Ad protein, including the p53-binding domains located around amino acid position 180 (Fig. 1*A*) and in the central part (4, 7, 8), plus two segments at the carboxy terminus that mediate inhibition of p53-dependent and p53-independent transactivation (5, 9, 10). Although the mechanism by which E1B-55kDa blocks transcription is still unclear, recent data suggest that its silencing activity requires a cellular corepressor that copurifies with RNA polymerase II (11) as well as interaction with cellular factors known to be involved in transcriptional repression such as histone deacetylase 1 (HDAC1) and mSin3A (12). Furthermore, consistent with its role in blocking p53-mediated transactivation, the Ad protein inhibits p53 acetylation by binding to both p53 and the transcriptional coactivator p300/CBP associated factor (PCAF; ref. 13).

Although neutralizing p53 tumor suppressor functions is an integral part of the molecular mechanisms used by E1B-55kDa to contribute to complete transformation of primary cells, it is now becoming clear that the mode of action of E1B-55kDa during transformation may involve additional functions and other protein interactions. In Ad subgroup C (Ad2 or Ad5) E1-transformed cells, most E1B-55kDa and p53 protein is excluded from the nucleus and colocalizes in cytoplasmic dense bodies that also contain the tumor suppressor protein WT1 and microfilaments (14, 15). This cytoplasmic restriction imposed on p53 and WT1 may involve continuous nucleocytoplasmic export mediated through a leucine-rich (NES) nuclear export signal located in the amino-terminal region of the 55-kDa polypeptide (ref. 16; Fig. 1*A*). Also, the E1B-associated protein E1B-AP5 and the nuclear bodies (NBs)-associated promyelocytic leukemia protein (PML) both can individually suppress transformation of primary rat cells by E1A plus E1B (17, 18), indicating that inhibition of these cellular factors by E1B may be required to enhance focus formation. Finally, Ornelles and Goodrum showed that E1B-55kDa relieves cell-cycle-imposed restrictions on viral replication through mechanisms independent of p53 (19, 20).

In light of these observations it was of considerable interest to identify further functional regions in the Ad5 E1B-55kDa protein that contribute to complete transformation of primary mammalian cells. In this study we demonstrate that Ad5 E1B-55kDa can be modified by the small ubiquitin-related modifier protein 1 (SUMO-1) *in vivo* and that SUMOylation is absolutely required to enhance focus formation in conjunction with E1A. Immunofluorescence analyses indicate that this posttranslational modification is required for efficient nuclear accumulation and intranuclear targeting of the Ad protein. These analyses introduce a new function for Ad5 E1B-55kDa and suggest that regulation of nuclear targeting is an important key to how the Ad protein contributes to transformation.

Materials and Methods

Plasmids and Transient Transfections. Unless stated otherwise, all plasmids used in this study use the cytomegalovirus major immediate early promoter from vector pcDNA3 to express Ad5 wild-type E1B-55kDa (pE1B-55K; ref. 21), the E1B mutant proteins R443 (pE1B-R443) and K104R (pE1B-K104R) V103D (pE1B-V103D), SUMO-1 (pSUMO-1) or an hemagglutinin (HA)-epitope-tagged SUMO-1 lacking amino acids 98–101 from the carboxy-terminus (pHA-SUMO-1, kindly provided by M. Nevels, Princeton University, Princeton). pE1B-R443 contains a 4-aa linker insertion at position 443 and was made by inserting the *Eco*RI/*Sal*I fragment from pGal4-R443 (9) into the *Eco*RI/*Xho*I sites of pcDNA3 (Invitrogen). pE1B-K104R carries a single amino acid change (lysine to arginine) at position 104 (K104R) that was introduced into pE1B-55K by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with

Abbreviations: Ad, adenovirus; BRK, baby rat kidney; E1B-55kDa, early region 1B 55-kDa oncoprotein; LMB, leptomycin B; NBs, nuclear bodies; SUMO-1, small ubiquitin-related modifier protein 1; HA, hemagglutinin; NES, nuclear export signal; PML, promyelocytic leukemia protein.

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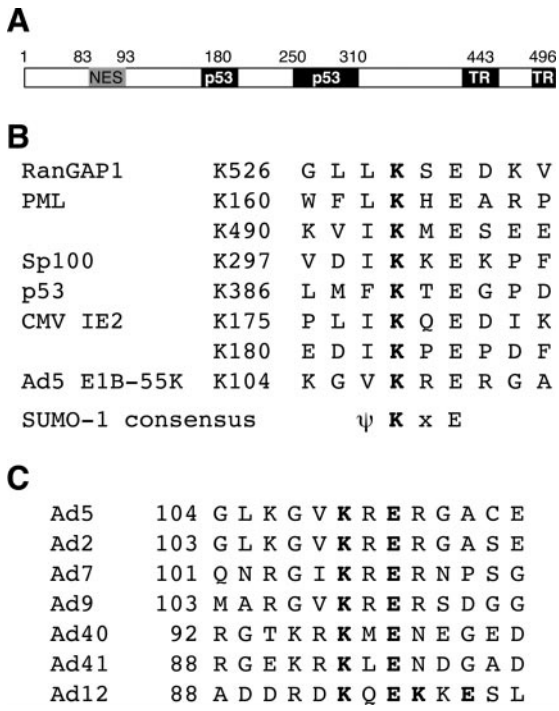

Fig. 1. (*A*) Diagram of Ad5 E1B-55kDa. Domains required for p53 binding (p53), transcriptional repression (TR), and transformation are indicated by black boxes. Numbers refer to amino acid residues, and 496 denotes the last amino acid. The location of the NES sequence is represented by a gray box. (*B*) Comparison of SUMO acceptor sites from several proteins with Ad5 E1B-55kDa. The consensus sequences of RanGAP1, PML, Sp100, p53, and cytomegalovirus (CMV) IE2 were derived from the sequences listed in ref. 28. Numbers indicate the position of the lysine residue (K) that serves as the SUMO attachment site; ψ stands for an aliphatic amino acid, and x represents any amino acid residue. (*C*) Alignment of potential SUMO acceptor sites in large E1B proteins from different human serotypes. Numbers indicate the position of the lysine residue (K) within the consensus motif.

synthetic oligonucleotide primers 484 5′-CGCGGCGGATCCCCACCATGTCTGACCAGGAGGC-3′ and 485 5′-GGCCGCGGATCCGAATTCCTAAACTGTTGAATGACC-3′. pE1B-V103D carries a single amino acid change (valine to aspartate) at position 103 (V103D) that was introduced into pE1B-55K with synthetic oligonucleotide primers 789 5′-GCTAAAGGGGGATAAGAGGGAGC-3′ and 790 5′-CGATTTCCCCCTATTCTCCCTCG-3′. To generate pSUMO-1, the *Bam*HI fragment of pGEX-SUMO-1 (generous gift of F. Melchior, Max Planck Institute, Martinsried, Germany) was cloned into the *Bam*HI site of pcDNA3. Plasmid pE1A contains the left end of the Ad5 genome (positions 1–1,916) including the *E1A* gene and its endogenous promoter. pC53-SN3 encodes human wild-type p53 from the pCMV/*neo* vector. The *firefly* luciferase reporter plasmid preLuc contains five p53-binding sites upstream of a minimal cytomegalovirus promoter and was obtained from N. Horikoshi, Washington University, St. Louis. Plasmids pGal4E1B-55kDa (22) and pGalTK-Luc (23) have been described previously. pGal4E1B-K104R and pGal4E1B-V103D were derived from pGal4E1B-55kDa by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the synthetic oligonucleotide primers 484, 485, 789, and 790 described above.

The p53-negative cell line H1299 (24) was grown in DMEM supplemented with 10% FCS. For dual luciferase assays, subconfluent H1299 cells were transfected as described previously (23) by using the indicated amounts of reporter and effector plasmids and 0.25 μg of pRL-TK (Promega), which expresses the *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter. Total cell extracts were prepared 36 h after transfection in lysis buffer, and *firefly* luciferase activity was assayed with 20 μl of extract. All samples were normalized for transfection efficiency by measuring *Renilla* luciferase activity.

Transformation Assays and Cell Lines. The generation of primary baby rat kidney (BRK) cells and BRK focus-forming assays were performed exactly as described previously (25). Three to four weeks after transfection, foci were stained with crystal violet (1% in 25% methanol) and dense foci of morphologically transformed cells were counted. To establish permanent cell lines, pools of foci were isolated and expanded in DMEM with 10% FCS plus 500 μg of G418 (Calbiochem) per ml. The transformed BRK cell line AB120 expresses the Ad5 E1A and wild-type Ad5 E1B-55kDa proteins. AB19 cells were established from foci obtained by cotransfection of pE1A and pE1B-K104R.

Protein Analysis. The following mAbs were used in this study: 2A6 is specific for E1B-55kDa (26), 5E10 is specific for PML (generously provided by L. de Jong, University of Amsterdam, The Netherlands), and the rat monoclonal antibody 9C10 is specific for Ad5 E1B-55kDa (kindly provided by A. Zantema, Leiden University, The Netherlands). Anti-HA mouse mAb 12CA5 and anti-HA rat mAb 3F10 were obtained from Roche (Gipf-Oberfrick, Switzerland). The anti-SUMO-1 mouse mAb 21C7 was from Zymed Laboratories.

For immunoprecipitation and/or immunoblotting, total cell extracts were prepared in RIPA assay buffer (50 mM Tris·chloride, pH 8.0/150 mM NaCl/0.1% SDS/1% Nonidet P-40/5 mM EDTA/0.5% sodium deoxycholate/0.1% Triton X-100) supplemented with a protease inhibitor mixture (Roche). After normalizing for protein concentration, whole-cell extracts were subjected to immunoprecipitation and/or immunoblotting exactly as described (18, 27). For testing SUMO-1 modification of E1B-55kDa *in vivo*, subconfluent H1299 cells were cotransfected with 4 μg of pE1B-55K or pE1B-K104R and 5 μg of pSUMO-1 or pHA-SUMO-1. Alternatively, MCF7 cells were infected with Ad5 *wt*300 virus at an infectivity of 20 plaque-forming units per cell. Total cell extracts were prepared 38 h after transfection or at the indicated time points after infection in RIPA assay buffer supplemented with 10 mM iodoacetamide (Sigma) and subjected to immunoblotting or combined immunoprecipitation/immunoblotting assays.

Immunofluorescence Microscopy. Indirect immunofluorescence analysis was performed as described previously (18). Cells grown on coverslips were fixed with paraformaldehyde (2% in PBS) for 10 min and reacted for 30 min with mAbs followed by 30 min incubation with the appropriate FITC- or Texas-red-conjugated secondary antibodies (Amersham Pharmacia). Coverslips were mounted in Glow medium (Energene, Regensburg, Germany), and images were acquired on a DMRB fluorescence microscope (Leica, Deerfield, IL) with a charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI).

Results

Ad5 E1B-55kDa Is Modified by SUMO-1 *in Vivo*. While mapping further domains in the Ad5 E1B-55kDa protein required for complete transformation of BRK cells, we identified several regions, including an amino-terminal segment from amino acid position 97 to 114 (C.E., unpublished data). Comparison with the protein database by using the BLAST homology finder revealed that amino acids 103 to 106 of this region (Fig. 1*B*) closely resemble the SUMO-1 attachment consensus sequence ΨKxE (reviewed in ref. 28), which also is conserved in the amino-terminal regions of large E1B proteins from human Ad serotypes 2, 7, and 9 (Fig. 1*C*). Although the large E1B proteins from

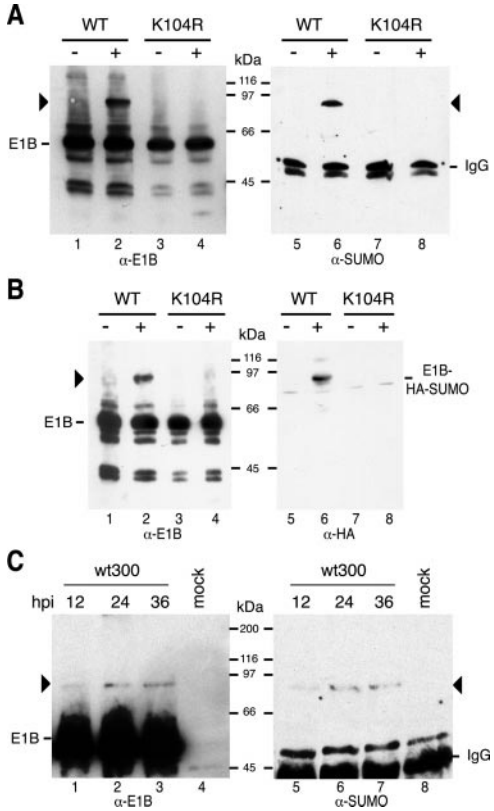

Fig. 2. Ad5 E1B-55kDa is covalently modified by SUMO-1 *in vivo*. (*A*) Subconfluent H1299 cells were transfected with pE1B-55K (WT) or pE1B-K104R (K104R) in the absence (−) or presence (+) of pSUMO-1. E1B-55kDa (E1B) and a slower-migrating form of the Ad protein indicated by black arrowheads were detected by the immunoblotting of total cell extracts with anti-E1B-55kDa mAb 2A6 (*Left*, α-E1B) or by immunoprecipitation of the same extracts with mAb 2A6 followed by immunoblotting with anti-SUMO-1 mAb 21C7 (*Right*, α-SUMO). (*B*) A similar high-molecular weight form of E1B-55kDa (*Left*, black arrowhead) was detected in total cell extracts prepared from H1299 cells transfected with pE1B-55K (WT) and pHA-SUMO-1 expressing an epitope-tagged SUMO-1 fusion protein. The high-molecular size band in lanes 2 and 6 corresponds to HA-SUMO-1-modified E1B-55kDa (E1B-HA-SUMO), as confirmed by Western blots with mAb 2A6 (*Left*, α-E1B) and anti-HA mouse mAb 12CA5 (*Right*, α-HA). (*C*) Covalent modification of E1B-55kDa by SUMO-1 in virus-infected cells. MCF7 cells were infected with *wt*300 virus, total cell extracts were prepared at the indicated time points in hours postinfection (hpi) and subjected to immunoprecipitation with mAb 2A6. Coprecipitated proteins were visualized by immunoblotting using mAbs 2A6 (*Left*, α-E1B) and 21C7 (*Right*, α-SUMO). E1B-55kDa (E1B) and the high-molecular weight form of E1B-55kDa (black arrowheads) are indicated. The positions of molecular mass markers are indicated in all panels.

Ad40, Ad41, and the highly oncogenic subgroup A virus Ad12 also contain a KxE consensus, they all lack the aliphatic amino acid residue (Ψ), which apparently is required for SUMO-1 modification (29). These findings prompted us to investigate whether Ad5 E1B-55kDa can be modified by SUMO-1. Plasmids encoding wild-type E1B-55kDa, SUMO-1, or epitope-tagged SUMO-1 (pHA-SUMO-1) were transfected into H1299 cells. In addition, we assayed two mutants of the 55-kDa protein each with a single mutation changing the aliphatic amino acid valine 103 to aspartate (E1B-V103D) and lysine 104 in the ΨKxE motif to arginine (E1B-K104R). Total cell extracts were prepared and analyzed by immunoblotting or combined immunoprecipitation/immunoblotting assays with the appropriate mAbs (Fig. 2 *A* and *B*). In cells transfected with only pE1B-55K expressing the wild-type Ad protein, a major band of 55 kDa corresponding to the Ad protein was detectable. In cells cotransfected with

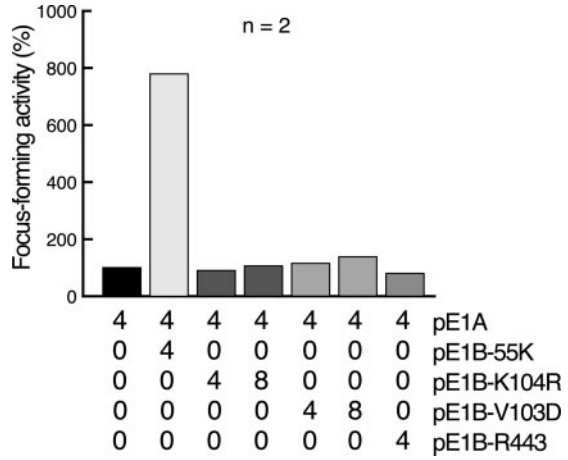

Fig. 3. Focus formation by Ad5 E1A, E1B-55kDa, E1B-K104R, E1B-V103D, and E1B-R443. Primary BRK cells were transfected with the indicated amounts of plasmids (micrograms of DNA per 3×10^6 cells). Focus-forming activity is represented as a percentage of E1A alone. The average number of foci for pE1A was 3 in two independent experiments.

pE1B-55K and pSUMO-1 or pHA-SUMO-1, an additional slower-migrating band of about 75 kDa corresponding to E1B-SUMO (Fig. 2*A*, lane 2) or E1B-HA-SUMO (Fig. 2*B*, lane 2) became visible. More importantly, the E1B-SUMO-modified forms were absent in cotransfections with the mutant pE1B-K104R and pSUMO-1 (Fig. 2*A*, lane 4) or the epitope-tagged SUMO-1 (Fig. 2*B*, lane 8). Similar results were obtained with pE1B-V103D and pHA-SUMO-1 (data not shown). These data clearly demonstrate that Ad5 E1B-55kDa can be SUMOylated *in vivo* and that the major SUMO-1 acceptor site in the Ad5 protein is the lysine residue at position 104 within the highly conserved ΨKxE motif. In the same assays, we reproducibly failed to detect SUMO-1-conjugated forms of the large E1B protein from Ad12 (data not shown), which indicates but does not prove that Ad12 E1B-54kDa is not a substrate of the SUMO-1 conjugation machinery. Finally, we assayed whether SUMO-1-modified forms of Ad5 E1B-55kDa exist in Ad-infected cells (Fig. 2*C*). The results from these experiments show that a slower-migrating form of E1B-55kDa of approximately 75 kDa coprecipitates with the Ad protein and corresponds to SUMO-1-modified E1B-55kDa as confirmed by Western blots with anti-E1B-55kDa and anti-SUMO-1 mAbs.

SUMO-1 Modification of Ad5 E1B-55kDa Is Required for Complete Transformation of BRK Cells and Inhibition of p53 Transactivation. To see what effect the K104R and V103D mutations have on the ability of E1B-55kDa to enhance focus formation in cooperation with E1A, we performed transformation assays with BRK cells (Fig. 3). We also assayed the effect of a previously described E1B mutant protein (E1B-R443), which carries a four-residue insertion at amino acid 443 (7). This protein binds to p53 with wild-type affinity but is defective in blocking p53 transcriptional activation, repression of transcription when tethered to DNA, and transformation (4, 9). Cotransfection of pE1A with plasmid pE1B-55K expressing the wild-type 55-kDa protein resulted in a more than 7-fold increase in foci compared with pE1A alone. By contrast, the E1B-K104R and E1B-V103D mutants were completely inactive for cooperative focus formation, similar to E1B-R443. Moreover, a 2-fold increase of pE1B-K104R or pE1B-V103D in the transfection mixture did not restore focus-forming activity. The few foci derived from transfections with pE1A and pE1B-K104R or pE1B-V103D developed more slowly and clearly differed in morphology from those with E1A plus

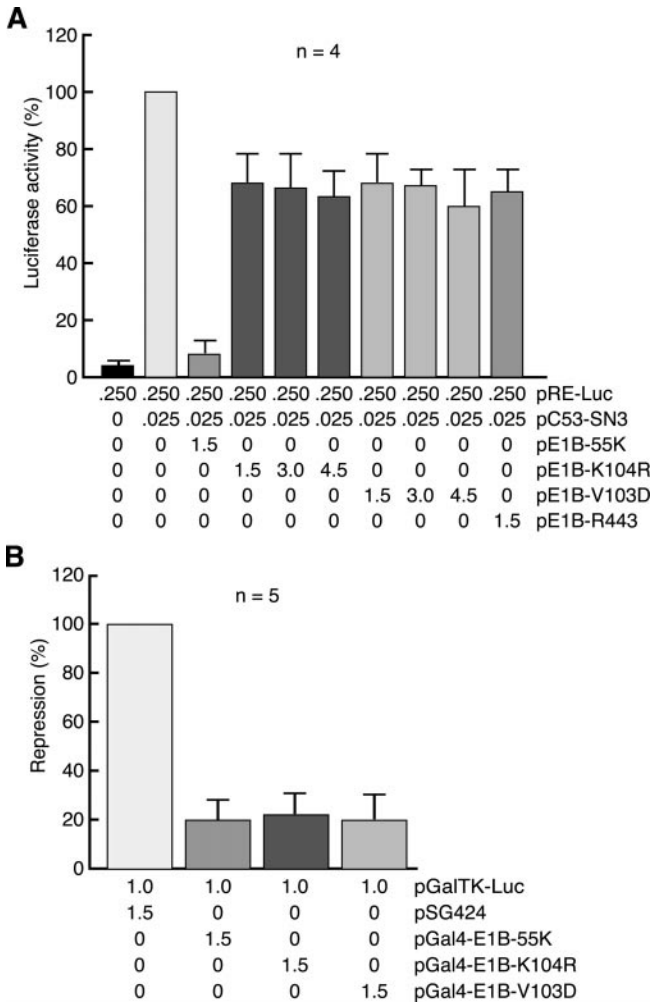

Fig. 4. Effects of E1B-K104R, E1B-V103D, and E1B-R443 on p53 transcriptional activation. (*A*) Subconfluent H1299 cells were transfected with the indicated amounts of reporter and effector plasmids (micrograms of DNA). The mean and standard deviation are presented for four experiments, each performed in duplicate. (*B*) Effect of E1B-K104R and E1B-V103D on transcriptional repression. H1299 cells were transfected with the indicated amount of effector and reporter plasmids (micrograms of DNA). The mean and standard deviation are presented for five experiments, each performed in duplicate. pSG424 denotes the parental plasmid expressing the Gal4 DNA-binding domain under the control of the simian virus 40 early promoter (37).

E1B-55K (data not shown), although they could be propagated into permanent cell lines after G418 selection.

Because complete transformation of BRK cells by E1A plus E1B correlates with the ability of the 55-kDa protein to function as a direct transcriptional repressor (4) that blocks transactivation of p53-responsive genes, we tested the effect of the K104R and V103D mutations on the protein's transcriptional properties. First, we asked whether E1B-K104R and E1B-V103D can inhibit p53 transcriptional activation of a cotransfected luciferase reporter plasmid containing five p53-binding sites in H1299 cells (Fig. 4*A*). As expected, wild-type E1B-55kDa blocked p53 induction of the reporter (pC53-SN3) approximately 10-fold, whereas cotransfection of increasing amounts of pE1B-K104R or pE1B-V103D reproducibly resulted in only a 20–40% reduction of luciferase activity, similar to E1B-R443. Next, we tested whether the K104R and V103D mutations interfere with the intrinsic repression activity of 55-kDa protein (Fig. 4*B*). The results from these assays show that cotransfection of plasmids encoding wild-type E1B-55kDa, K104R and V103D fused to the Gal4 DNA-binding domain, which contains a potent nuclear localization signal, efficiently repressed transcription from the herpes simplex virus thymidine kinase promoter with five upstream Gal4-binding sites. Thus, the K104R and V103D mutations substantially impair the ability of E1B-55kDa to inhibit p53 transactivation but not its ability to function as a direct transcriptional repressor when tethered to the herpes simplex virus thymidine kinase promoter. These results, together with the finding that p53 specifically coprecipitates with the mutant proteins (data not shown), demonstrate that E1B-K104R and E1B-V103D can bind to p53 but are defective for both inhibition of p53 transactivation function and transformation. Apparently these activities depend on the presence of valine at position 103 and lysine at 104, the principle SUMO-1 acceptor site in the Ad protein.

SUMO-1 Modification Affects the Subcellular Localization of E1B-55kDa. Previous studies with subgroup C (Ad2 or Ad5) E1-transformed human and rodent cells have shown that E1B-55kDa sequesters p53 along with WT1 in a cytoplasmic body (14, 15). The cytoplasmic restriction imposed on both tumor suppressor proteins may involve continuous nuclear export of a trimeric E1B/p53/WT1 protein complex (15) mediated by the recently identified NES in the E1B protein (16). Also, evidence is accumulating for a role of SUMO-1 in the regulation of protein targeting to specific subcellular sites (reviewed in ref. 28). We therefore wondered whether SUMO-1 modification might modulate the subcellular localization of the Ad protein. To test the subcellular localization of the Ad protein, we performed indirect immunofluorescence analyses (Fig. 5) with transformed BRK cells stably expressing wild-type E1B-55kDa (AB120 cells) or the E1B-K104R mutant protein (AB19 cells). In AB120 (Fig. 5 *Aa–Ac*) and AB19 cells (Fig. 5 *Ad–Af*) most E1B-55kDa localized in a large cytoplasmic body (Fig. 5 *Aa* and *Ad*), whereas SUMO-1 exhibited a dominant diffuse nuclear distribution in both cell lines (Fig. 5 *Ab* and *Ae*). The subcellular localization of wild-type E1B-55kDa, however, was dramatically changed when we transfected a plasmid expressing the epitope-tagged HA-SUMO-1 fusion protein (Fig. 5*B*). In these cells, wild-type E1B-55kDa was directed to the nucleus where it colocalized with HA-SUMO-1 in either track-like (Fig. 5 *Ba–Bc*) or dot-like structures (Fig. 5 *Bd–Bf*). By contrast, no apparent effect on the localization of the Ad protein was observed when HA-SUMO-1 was expressed in AB19 cells containing the E1B-K104R mutant protein. In these cells, most E1B-K104R was excluded from the nucleus and still sequestered in cytoplasmic bodies closely attached to the nuclear membrane (Fig. 5 *Bg–Bi*). The SUMO-1-dependent localization of the wild-type but not the mutant protein to distinct nuclear sites suggested that this modification may modulate the intranuclear targeting of the Ad protein on its entry into the nucleus. Alternatively, these findings may implicate that SUMOylation additionally regulates nuclear import of E1B-55kDa.

To test the latter possibility, we took advantage of the fact that the majority of cytoplasmic E1B-55kDa is relocalized to the nucleus when cells are treated with leptomycin B (LMB) (16), an inhibitor of the chromosome region maintenance 1 (CRM1)-dependent nuclear export of leucine-rich NES-containing proteins (reviewed in ref. 30). Thus, if the K104R mutant protein is defective for nuclear transport it should not be able to accumulate in the nucleus in the presence of LMB. Consistent with this model, wild-type E1B-55kDa was redistributed to the nucleus where it accumulated in large aggregates that also partially contained the endogenous SUMO-1 protein (Fig. 6 *a–c*). By contrast, most of the mutant protein was excluded from the nucleus and remained mostly concentrated in cytoplasmic bodies (Fig. 6 *d* and *e*), indicating that the K104R mutation substantially impairs the ability of the Ad protein to translocate to the nucleus.

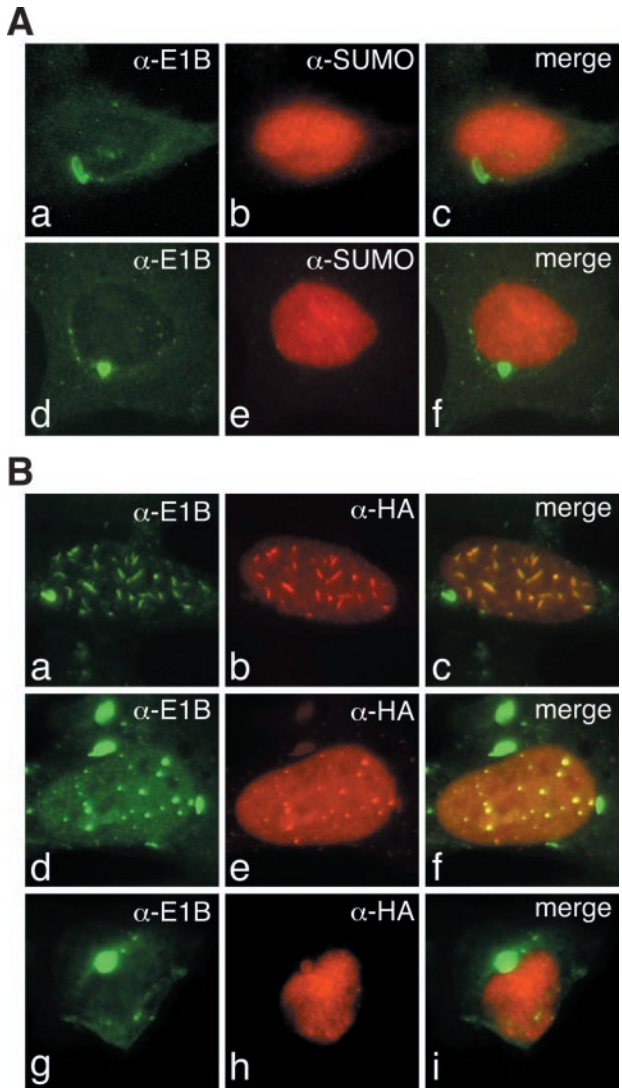

Fig. 5. (*A*) Subcellular localization of E1B-55kDa and SUMO-1 in transformed rat cells. AB120 and AB19 cells expressing wild-type E1B-55kDa (*a–c*) or the K104R mutant protein (*d–f*), respectively, were double-labeled *in situ* with anti-E1B-55kDa rat mAb 9C10 or mouse mAb 21C7 specific for SUMO-1. These were detected with FITC- and Texas-red-conjugated secondary antibodies, respectively. Anti-E1B (green, *a* and *d*) and anti-SUMO-1 (red, *b* and *e*) staining patterns are shown. An overlay of these two patterns (merge) is shown in *c* and *f*. (*B*) Effect of ectopically expressed HA-SUMO-1 on the localization of wild-type E1B-55kDa and E1B-K104R. AB120 (*a–f*) and AB19 cells (*g–i*) were transfected with 4 μg pHA-SUMO-1. After 36 h, cells were double-labeled with anti-E1B-55kDa mAb 2A6 (green, *a*, *d*, and *g*) and anti-HA rat mAb 3F10 (red, *b*, *e*, and *h*). The overlay of these patterns (merge) are shown in *c*, *f*, and *i*. (×7,600.)

On the basis of these results we conclude that covalent modification of lysine 104 by SUMO-1 is required for both efficient nuclear accumulation and intranuclear targeting to specific sites.

Discussion

During the past few years, several viral and cellular proteins have been reported to be covalently modified by SUMO-1 (reviewed in ref. 28). Extensive evidence suggests that this posttranslational modification regulates protein–protein interactions and/or subcellular localization. Several of the verified and potential SUMO-1 substrates have been implicated in DNA replication, cell cycle control, apoptosis, and signal transduction, indicating that SUMOylation plays a role in various aspects of cell growth

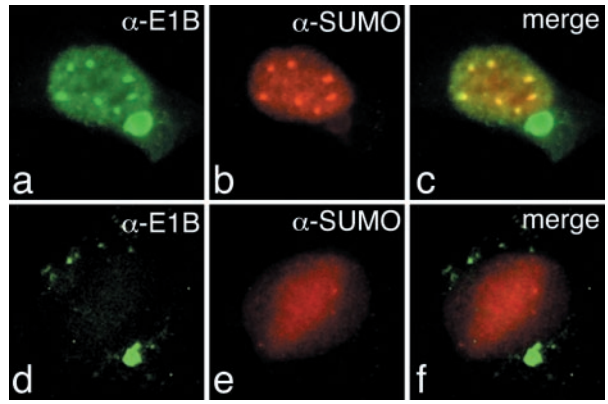

Fig. 6. Subcellular localization of E1B-55kDa and SUMO-1 in the presence of LMB. AB120 (*a–c*) and AB19 cells (*d–e*) were grown for 3 h in medium containing 10 nM LMB before fixation. Then the cells were double-labeled with mAbs 9C10 (green, *a* and *d*) and 21C7 (red, *b* and *e*). The overlay of these images (merge) is shown in *c* and *f*. (×7,600.)

control. In this study we demonstrate that the Ad5 E1B-55kDa oncoprotein can be covalently modified by SUMO-1 at a single lysine residue (K104) embedded in the SUMO-1 attachment consensus motif ΨKxE. This demonstration correlates well with the recent finding that a peptide corresponding to amino acids 99 to 109 of the Ad5 E1B-55kDa protein is efficiently SUMOylated at K104 in an *in vitro* assay with purified components (29). These results strongly suggest that Ad5 E1B-55kDa is a substrate of the SUMO-1 conjugation system in intact cells as well as *in vitro*.

Complete transformation of primary rat cells by E1A and E1B correlates with the ability of the 55-kDa protein to bind to p53 and to block p53-mediated transcriptional activation (5, 9, 10). Apparently, the latter activity is regulated by SUMO-1 conjugation on lysine 104, because the E1B-K104R and E1B-V103D mutant proteins are defective for both inhibition of p53 transactivation function (Fig. 4) and cooperative focus formation (Fig. 3). Our data fit well in a model in which SUMO-1 modification regulates nuclear accumulation and/or targeting of the Ad5 oncoprotein to specific nuclear sites. In contrast to cotransfection experiments in which substantial amounts of E1B-SUMO-1-conjugated forms were detected by Western blot analysis (Fig. 2), only a small percentage of E1B-55kDa protein can be detected as a conjugate with SUMO-1 in lytically infected human cells (Fig. 2) and transformed BRK cells (C.E., unpublished data). One possible explanation for this result is that the modification is transient and that the ratio between the modified and unmodified forms reflects the different subcellular localizations of free nuclear SUMO-1 and the cytoplasmic E1B-55kDa target protein, which shuttles rapidly between both compartments. The fraction of the conjugated form can be increased when SUMO-1 is overexpressed in the transformed rat cells. In these cells, a large portion of wild-type E1B-55kDa but not E1B-K104R accumulates in the nucleus where it colocalizes with SUMO-1 in track- or dot-like structures (Fig. 5), which indicates that SUMO-1 conjugation is required for efficient nuclear accumulation of the Ad protein. Conversely, SUMO-1 deconjugation may be important for its efficient nuclear export mediated by the NES. Such a mechanism is compatible with the observation that blocking E1B-55kDa export by LMB results in the efficient nuclear accumulation of the wild-type but not the K104R mutant protein (Fig. 6), and provides an explanation for the finding that the mutant protein is severely defective in inhibition of p53 transactivation in transient transfection assays (Fig. 4). The cytoplasmic restriction imposed on E1B-K104R obviously can be overcome when the mutant protein is fused to

the amino-terminal 147 aa of the Gal4 protein that include a potent nuclear localization signal (Fig. 4). Apparently, in the presence of the Gal4 DNA-binding domain, sufficient E1B-K104R can enter the nucleus to repress transcription of the herpes simplex virus thymidine kinase reporter.

Our data additionally suggest that SUMO-1 modification may play a role in the intranuclear targeting of the Ad protein to specific sites. In transfected cells expressing an epitope-tagged SUMO-1 protein, E1B-55kDa colocalized with SUMO-1 in track- or dot-like structures (Fig. 5) that closely resembled reorganized NBs previously described for Ad-infected (31, 32) or transformed BRK cells stably expressing E1A/E1B and the E4orf3 protein (18). Recent studies indicate that the NB-associated marker protein PML physically interacts with p53, regulates p53 acetylation, and activates p53 tumor suppressor function, which seems to depend on the localization of both proteins in NBs (33–35). Although clearly speculative, these findings, along with the results presented in this report, hint at the possibility that inhibition of p53 transactivation by E1B-55kDa may occur at the NBs. This possibility is intriguing because the colocalization of E1B-55kDa with NBs in virus-infected cells suggests that the E1B protein can interact with components of the NBs (31). The observation that modulation of PML functions by E1B proteins may be required for complete transformation of BRK cells in cooperation with E1A (18) provides further support for this view.

In conclusion, we show here that SUMO-1 modification regulates an essential activity of the E1B-55kDa oncoprotein, most probably nuclear targeting. Based on the finding that SUMO-1-conjugated forms of E1B-55kDa exist in Ad-infected cells (Fig. 2), it is almost certain that this modification plays an important role in the regulation of its multiple lytic functions. Because two proteins of early region 4 (E4orf3 and E4orf6) have been shown to regulate nuclear accumulation and targeting into reorganized NB structures and nuclear export of E1B-55kDa (reviewed in ref. 36), it is possible that both E4 proteins may modulate SUMO-1 conjugation and deconjugation in productively infected cells. The further characterization of these processes will clarify pathways modulated by the E1B and E4 oncoproteins and, perhaps, reveal new principles of viral transformation relevant to human neoplasms.

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1. Shenk, T. (1996) in *Virology*, eds. Fields, B. N., Knipe, D. M. & Howley, P. M. (Lippincott-Raven, New York), Vol. 2, pp. 2111–2148.
2. Barker, D. D. & Berk, A. J. (1987) *Virology* **156,** 107–121.
3. Sarnow, P., Ho, Y. S., Williams, J. & Levine, A. J. (1982) *Cell* **28,** 387–394.
4. Yew, P. R. & Berk, A. J. (1992) *Nature (London)* **357,** 82–85.
5. Teodoro, J. G. & Branton, P. E. (1997) *J. Virol.* **71,** 3620–3627.
6. Hutton, F. G., Turnell, A. S., Gallimore, P. H. & Grand, R. J. (2000) *Oncogene* **19,** 452–462.
7. Yew, P. R., Kao, C. C. & Berk, A. J. (1990) *Virology* **179,** 795–805.
8. Grand, R. J., Parkhill, J., Szestak, T., Rookes, S. M., Roberts, S. & Gallimore, P. H. (1999) *Oncogene* **18,** 955–965.
9. Yew, P. R., Liu, X. & Berk, A. J. (1994) *Genes Dev.* **8,** 190–202.
10. Teodoro, J. G., Halliday, T., Whalen, S. G., Takayesu, D., Graham, F. L. & Branton, P. E. (1994) *J. Virol.* **68,** 776–786.
11. Martin, M. E. & Berk, A. J. (1999) *Mol. Cell. Biol.* **19,** 3403–3414.
12. Punga, T. & Akusjarvi, G. (2000) *FEBS Lett.* **476,** 248–252.
13. Liu, Y., Colosimo, A. L., Yang, X. J. & Liao, D. (2000) *Mol. Cell. Biol.* **20,** 5540–5553.
14. Zantema, A., Fransen, J. A., Davis, O. A., Ramaekers, F. C., Vooijs, G. P., DeLeys, B. & van der Eb, A. J. (1985) *Virology* **142,** 44–58.
15. Maheswaran, S., Englert, C., Lee, S. B., Ezzel, R. M., Settleman, J. & Haber, D. A. (1998) *Oncogene* **16,** 2041–2050.
16. Krätzer, F., Rosorius, O., Heger, P., Hirschmann, N., Dobner, T., Hauber, J. & Stauber, R. H. (2000) *Oncogene* **19,** 850–857.
17. Gabler, S., Schütt, H., Groitl, P., Wolf, H., Shenk, T. & Dobner, T. (1998) *J. Virol.* **72,** 7960–7971.
18. Nevels, M., Täuber, B., Kremmer, E., Spruss, T., Wolf, H. & Dobner, T. (1999) *J. Virol.* **73,** 1591–1600.
19. Goodrum, F. A. & Ornelles, D. A. (1997) *J. Virol.* **71,** 548–561.
20. Goodrum, F. D. & Ornelles, D. A. (1998) *J. Virol.* **72,** 9479–9490.
21. Nevels, M., Täuber, B., Spruss, T., Wolf, H. & Dobner, T. (2001) *J. Virol.* **75,** 3089–3094.
22. Dobner, T., Horikoshi, N., Rubenwolf, S. & Shenk, T. (1996) *Science* **272,** 1470–1473.
23. Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H. & Dobner, T. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1206–1211.
24. Mitsudomi, T., Steinberg, S. M., Nau, M. M., Carbone, D., D'Amico, D., Bodner, H. K., Oie, H. K., Linnoila, R. I., Mulshine, J. L., Minna, J. D., *et al.* (1992) *Oncogene* **7,** 171–180.
25. Nevels, M., Spruss, T., Wolf, H. & Dobner, T. (1999) *Oncogene* **18,** 9–17.
26. Sarnow, P., Sullivan, C. A. & Levine, A. J. (1982) *Virology* **120,** 510–517.
27. Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H. & Dobner, T. (2000) *J. Virol.* **74,** 5168–5181.
28. Melchior, F. (2000) *Annu. Rev. Cell. Biol.* **16,** 591–626.
29. Rodriguez, M. S., Dargemont, C. & Hay, R. T. (2000) *J. Biol. Chem.* **276,** 12654–12659.
30. Görlich, D. & Kutay, U. (1999) *Annu. Rev. Cell. Dev. Biol.* **15,** 607–660.
31. Doucas, V., Ishov, A. M., Romo, A., Juguilon, H., Weitzman, M. D., Evans, R. M. & Maul, G. G. (1996) *Genes Dev.* **10,** 196–207.
32. Carvalho, T., Seeler, J. S., Ohman, K., Jordan, P., Pettersson, U., Akusjärvi, G., Carmo Fonseca, M. & Dejean, A. (1995) *J. Cell. Biol.* **131,** 45–56.
33. Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P. P., Will, H., Schneider, C. & Del Sal, G. (2000) *EMBO J.* **19,** 6185–6195.
34. Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W. & Paolo Pandolfi, P. (2000) *Nat. Cell. Biol.* **2,** 730–736.
35. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., *et al.* (2000) *Nature (London)* **406,** 207–210.
36. Dobner, T. & Kzhyshkowska, J. (2001) *Curr. Top. Microbiol. Immunol.* **259,** 25–54.
37. Sadowski, I. & Ptashne, M. (1989) *Nucleic Acids Res.* **17,** 7539.