New Insights into BS69 Functions

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The BS69 protein has been commonly described as a co-repressor associated with various transcription factors. However, this hypothesis relied predominately on overexpression of tagged proteins due to the lack of a reliable BS69 antibody. We present for the first time a complete sequence of BS69 and valuable tools to characterize the endogenous protein. We show that the full-length BS69 protein, as well as minor alternatively spliced isoforms, is ubiquitously expressed, nuclear, and associates with chromatin and mitotic chromosomes. Accordingly, BS69 interacts with a set of chromatin remodeling factors, including ATP-dependent helicases, histone deacetylases, and histone methyltransferases, as well as the E2F6 transcription factor. These data strengthen a role for BS69 in gene repression and link BS69 to chromatin remodeling.

BS69 was originally defined as a cellular protein targeted by the adenoviral E1A and the Epstein-Barr viral EBNA2 proteins (1, 2). Further analysis revealed that BS69, via its cysteine-rich MYND domain, recognizes a similar PXXXP peptide motif present in both viral proteins as well as in cellular partners. In vitro, binding of viral proteins to BS69 led to the disruption of the cellular BS69-associated complexes (2). Despite its potential tumor suppressor gene properties, the function of BS69 remains elusive. The protein was found to associate with various transcription factors such as c-Myb, B-Myb, MGA, and Ets-2 as well as the EMSY protein, a recently identified BRCA2 negative regulator (3–6). In keeping with its potential role in gene repression, we further investigated the BS69 in vivo functions was hindered by the lack of reliable antibody and functional tests.

The results presented herein clearly show that the main product of the BS69 gene is a ubiquitous protein of 74 kDa, with three additional minor truncated isoforms generated through alternative splicing mechanisms. All four products were found to be unstable, subject to degradation through the ubiquitin-proteasome degradation pathway, nuclear, and associated with chromatin and mitotic chromosomes. In keeping with its potential role in gene repression, we further found that the endogenous full-length BS69 protein immunoprecipitated a set of chromatin remodeling factors.

EXPERIMENTAL PROCEDURES

Constructs—The human BS69 cDNAs were obtained by reverse transcription-PCR. First strand cDNA was synthesized from total RNA extracted from IMR90 human primary fibroblasts using Superscript™ II (Invitrogen) and oligo(dT) (Invitrogen). PCR was performed with the primer pairs 1 + 2 (for the variants encompassing a MYND domain) and 1 + 3 (for the MYND-less variants) by means of 25 cycles at an annealing temperature of 55 °C. PCR products were subcloned into the pRC-CMV or into the FLAG-pcDNA3 expression vectors (12).

The sequences of the primers used are as follows: primer 1, GCGAAT-TCGACGTTTAAACAAAGACGACAGCCGATAc; primer 2, CGGAATTCGTCATCTTTCGCGCGACGGTGCGC; primer 3, GCGAATTCGTCGTCATGCGACGTCACGTCCTTC. cDNA sequences are accessible in the GenBank™ library under the accession numbers DQ335452, -3, -4, and -5. BS69 deletion mutants and BS69 fusion proteins (GFPmut1 variant, Gal4DNA-binding domain (amino acids 1–147)) were generated by PCR and cloned into the vertebrate expression vector pcDNA3 or FLAG-pcDNA3. BS69-GST fusion proteins have been described previously (2). The shRNA directed against the BS69 RNA was designed using the target sequence CCG-GATGAAAGCCTGACCAA. A shRNA directed against the luciferase GL3 sequence CTTCACGCTGATCTCTCGA was used as a control. Oligonucleotides were subcloned into the pRS expression vector (13) using an optimized loop according to (14). HA-tagged Brahma, Myc-tagged HDAC-1, Myc-tagged EZH1/2, HA-tagged E2F6, and HA-tagged ubiquitin expression vectors were generously provided by Christian Muschardt, Bernard Luscher, Susanne Opravil, Stefan Gaubatz, and Samuel Buchbaum, respectively.

Antibodies—A peptide encompassing the residues 358–441 of the human BS69 protein (GenBank™ accession number X86098) was expressed in bacteria as a GST fusion protein, purified by affinity chromatography on glutathione beads, and used as immunogen in rabbits. The resultant polyclonal antibody was purified by immuno-affinity using the GST orientation kit (Pierce).

Western blotting and immunoprecipitations were performed with a polyclonal α-c-Myc A14 antibody (tebu-bio), a monoclonal α-FLAG M2 (Stratagene), a monoclonal α-HA.11 (Babco), goat polyclonal α-BRG1 N-15 and α-HDAC-1 C-19 antibodies (tebu-bio), a mouse monoclonal α-EZH2 M10 antibody (15), a rabbit polyclonal α-RACK7 antibody,7 and a rabbit polyclonal α-E2F6 antibody directed against the C-terminal peptide CEEPNQQSEELVSN of the human E2F6 protein.8 Non-immune IgG was purchased by Sigma.

Cell Culture—Murine and human cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum, 10 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C. The quail fibroblasts QT6 were cultured in Dulbecco’s modified Eagle’s medium containing 8% fetal calf serum and 2% chicken serum, 10 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin at 39 °C. For inhibition of the proteasome, MG132 (5 mM) was added to the medium containing 8% fetal calf serum and 2% chicken serum, 10 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin at 39 °C. For inhibition of the proteasome, MG132 (5 μM) for QT6 fibroblasts; 10 μM for 293T, Calbiochem) and epoxomicin (2 μM, Calbiochem) were added to the medium.

The abbreviations used are: GST, glutathione S-transferase; shRNA, small hairpin RNA; HA, hemagglutinin; GFP, green fluorescent protein; EGFP, enhanced GFP.

8 Non-immune IgG was purchased by Sigma.

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**Pulse-Chase Experiments**—HeLa cells (8 × 10⁵) were transiently transfected with 2 μg of full-length BS69 pRC-CMV expression vector. Cells were incubated with a methionine- and cysteine-free medium (Invitrogen) for 45 min, pulse-labeled with [³⁵S]methionine and cysteine (50 μCi/ml cell-labeling Promix, Amersham Biosciences) for 1 h and chased for different times in the presence of 10 μM MG132 (or Me₂SO). BS69 expression was examined by immuno-precipitation using 2 μg of BS69 polyclonal antibody.

**In Vivo Ubiquitination Assays**—In vivo ubiquitination assays were performed as described (16). Briefly, 293T cells (2 × 10⁶) were transiently co-transfected with 0.5 μg of the full-length BS69 expression vector and an increasing amount of the HA-ubiquitin pTL1 expression vector. 24 h post-transfection, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS) supplemented with 5 mM N-ethylmaleimide and protease inhibitors, sonicated, and cleared by centrifugation. Equal amounts of proteins were immunoprecipitated with 2 μg of α-BS69 antibody and incubated with protein A-Sepharose beads. Bead washes were performed in RIPA buffer. Ubiquitinated-BS69 proteins were visualized by Western blotting with a monoclonal antibody (Babco).

**Cell Fractionation Assays**—293T cells (5 × 10⁵) were transfected with 2 μg of BS69 expression vectors. Fractionation assays were performed according to Ref. 17.

**BS69 Knockdown and Immunofluorescence**—293T cells were transiently transfected with 5 μg of BS69-shRNA or luc-shRNA in pRS and maintained 3 days under selection. Cells were fixed in a 4% paraformaldehyde solution, permeabilized in a 50 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween 20 (TBST) buffer supplemented with 0.4% Triton X-100 for 10 min. Cells were successively blocked in a TBST, 3% bovine serum albumin, 2% gelatin buffer for 30 min, incubated with the α-BS69 antibody (3.6 μg/ml in blocking solution), washed with TBST, incubated with a rhodamine-conjugated anti-rabbit antibody (1/2500, Jackson ImmunoResearch Laboratories), washed in TBST, and stained with 4',6-diamidino-2-phenylindole.

In experiments employing the GFP fusion proteins, cells were similarly fixed, permeabilized and stained with 4',6-diamidino-2-phenylindole. Images were observed with an axioplan 2 microscope (Zeiss).

**Protein Interaction Analysis Co-immunoprecipitation Experiments and GST Pulldown Assays**—QT6 fibroblasts (3 × 10⁶) were transfected with 3 μg of each expression vector. Cells were successively lysed in a 100 mM NaCl, 20 mM Tris, pH 8, 0.5% Nonidet P-40 buffer supplemented with protease inhibitors, sonicated, and cleared by centrifugation. Lysates were immunoprecipitated either with 3 μg of the monoclonal α-HA (Babco), or 2 μg of a polyclonal α-Myc antibody (tebu-bio) at 4°C for 4 h. Protein A-Sepharose (Amersham Biosciences) was added and incubated at 4°C for 1 h. Beads were washed in extraction buffer, and proteins were separated by SDS-PAGE. Immunoprecipitations employing the endogenous BS69, RACK7, EZH2, BRG1, HDAC1, and E2F6 proteins were performed from 10⁷ 293 cells. Nuclear extracts, generated as described in Ref. 17, were lysed in a 100 mM NaCl, 30 mM Tris, pH 7.5, EDTA (1 mM for BS69 immunoprecipitation and 10 mM for RACK7 immunoprecipitation), 1 mM dithiothreitol, 0.4% Triton X-100, 4% glycerol extraction buffer supplemented with protease inhibitors. Lysates were immunoprecipitated with 2 μg of α-BS69 or 5 μg of α-RACK7 antibody at room temperature for 2 h. GST pulldown assays were performed as described in Ref. 2.

**RESULTS**

**Analysis of the BS69 Products**—A comparison of the published BS69 sequences (1) with the data base entries revealed a disparity in the 5’ extremity potentially encoding for forty additional amino acids. The extra sequences perfectly map to all expressed sequence tag sequences available and are extremely well conserved during evolution (Fig. 1). To clarify this point, BS69 was cloned by PCR using primers corresponding to both proposed sequences and ectopically expressed. Indeed, comparison of the cDNA translation products with the endogenous protein by SDS-PAGE confirmed that the additional sequences are included in the protein (Fig. 1B). Knockdown of endogenous BS69 by small interfering RNA further confirmed the identity of the 74-kDa band (Fig. 1B).

We also identified variants of the BS69 protein (Fig. 1A). The alternative splicing of exon III gives rise to an additional RNA encoding for a protein lacking the PHD domain. The boundaries and the alternative splicing of the exon are conserved in murine species. A MYND-less variant of BS69 has also been described (GenBank™ accession number NM212479). Using appropriate primers, we confirmed that the RNA encoding for such a variant exits and results from the alternative usage of the exon XII splice donor. Cloning of an isoform lacking both the PHD and MYND domains further indicated that the alternative splicing of exon III and XII are independent. In primary fibroblasts and in all cell lines tested, the PHD- and MYND-less variants are largely under-represented (Fig. 1C and data not shown). The full-length BS69 is therefore the main BS69 isoform expressed. Moreover, we failed to amplify any
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FIGURE 2. BS69 isoforms are subject to the ubiquitin-dependent proteasome degradation pathway. A, the treatment of cells with proteasome inhibitors extends the half-life of BS69. HeLa cells, transiently transfected with a full-length BS69 expression vector, were labeled with [35S]methionine/cysteine mixture for 1 h and chased for various periods of time, as indicated on top, in the presence of MG132 or MeSO as a control. BS69 proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. B, accumulation of BS69 in the presence of proteasome inhibitors. 293T cells were untransfected (left side) or transiently transfected with a full-length BS69 expression vector (right side). After 24 h, cells were either treated with MG132 (MG), epoxomicin (Ep), or dimethyl sulfoxide (DMSO) as a control. Endogenous (endo) or ectopically expressed (trans) BS69 protein expression was examined by Western blotting. C, the human full-length BS69 is polyubiquitinated. 293T cells were transiently transfected with various amounts of HA-tagged ubiquitin (HA-Ubi) expression vectors as indicated on top. Endogenous (endo, left panel) or ectopically expressed (trans, right panel) BS69 proteins were immunoprecipitated. BS69 expression control (bottom) and ubiquitylated BS69 proteins (top) were examined by Western blotting using α-BU69 and α-HA antibodies, respectively. D, all BS69 isoforms similarly accumulate in the presence of proteasome inhibitors. 293T cells were transiently transfected with BS69 expression vectors as indicated on left. Cells were either left untreated or were treated for 10 h with epoxomicin (Ep). Protein expression was examined by Western blotting using an α-BU69 antibody. E, all BS69 isoforms are similarly subject to polyubiquitination. 293T cells were transiently co-transfected with HA-ubiquitin (HA-Ubi) and BS69 expression vectors as indicated on top. Ubiquitylation of each isoform was analyzed as indicated for C. The ladder of ubiquitylation observed with the endogenous protein after a long exposure is presented on the right side. F, the BS69 elements regulating stability are located in the N-terminal half of the protein. As schematically represented on the left, BS69 peptides or BS69-Gal4DBD fusion proteins, all of which were FLAG-tagged, were transiently transfected in Q76 fibroblasts. 24 h later, cells were either left untreated or treated with MG132 for 10 h. Protein expression was examined by Western blotting using a monoclonal α-FLAG M2 antibody.

BRAM1 cDNA, a product previously described as an additional BS69 splice variant (18).

BS69 is Subject to the Ubiquitin Proteasome Degradation Pathway—The additional BS69 N-terminal sequences prompted us to re-examine the properties of the protein. We first estimated the half-life of the full-length protein using pulse-chase experiments. In HeLa and 293T cells, its half-life was found to be restricted to 3 h but could be further extended after treatment of cells with MG132, a drug commonly used as a proteasome inhibitor (Fig. 2A and data not shown). To confirm that BS69 is subject to degradation through the proteasome pathway, we utilized epoxomicin, one of a novel generation of drugs known to strictly target the proteasome (19). We found that both MG132 and epoxomicin similarly led to the accumulation of endogenous or ectopically expressed proteins (Fig. 2B). We then tested whether BS69 undergoes ubiquitylation. 293T cells were transiently co-transfected with full-length BS69 and HA-tagged ubiquitin expression vectors. When BS69 proteins were immunoprecipitated and blotted with an anti-HA antibody, a typical ladder of ubiquitylated proteins was detected, in a HA-ubiquitin dose-dependent manner (Fig. 2C). Similar results were obtained with the endogenous full-length BS69 protein (Fig. 2C). These experiments were next repeated with the other splice variants, all of which were found to accumulate in the presence of proteasome inhibitors and be subject to ubiquitylation (Fig. 2, D and E). We therefore concluded that the stability of all four BS69 splice variants is similarly regulated. To identify the residues that control protein stability, truncated BS69 peptides were generated and transiently transfected in fibroblasts, either left untreated or treated with MG132. As for the full-length protein, a peptide encompassing the N-terminal half of the protein was found to be unstable unless expressed in the presence of MG132. Inversely, deletion of these same N-terminal sequences was sufficient to generate a stable peptide. Furthermore, the fusion of the same sequences to the Gal4 DNA-binding domain, or to the GFP protein, provided instability (Fig. 2F and data not shown). Thus, we concluded that the N-terminal half of the BS69 protein regulates protein stability and that these properties are transferable. As a control, the fusion of the C-terminal peptide to the same proteins did not affect their stability (Fig. 2F). We failed to further map by deletion analysis the BS69 sequences implied in protein stability, suggesting potentially that multiple lysine residues distributed along the N terminus are ubiquitylated.

BS69 Proteins Recruit Chromatin-remodeling Factors—To gain an insight into BS69 functions we examined the sublocalization of the endogenous BS69 protein by immunofluorescence. A homogenous nuclear staining, the specificity of which was confirmed by RNA interference, was observed in HeLa and 293T cells (Fig. 3A). A similar staining was observed with transient transfection of the various BS69 isoforms (Fig. 3B). We further investigated BS69 localization in a cell fractionation assay. The endogenous protein, as well as each of the ectopically expressed BS69 isoforms, was strictly found to associate with the insoluble chromatin-nuclear matrix fraction. The prior treatment of nuclei with micrococccus nuclease completely solubilized all isoforms. Thus, we concluded that BS69 proteins specifically associated with chromatin and not the nuclear matrix (Fig. 3C). We next analyzed whether BS69 associates with mitotic chromosome. A full-length BS69-GFP fusion protein (FL-GFP) was generated and transiently transfected in 293T and HeLa cells. Whereas the protein was uniformly spread through the nucleoplasm in interphase cells, it was found to associate with condensed chromosomes during mitosis. In comparison, a GFP fusion protein encompassing the N-terminal two-thirds of the protein (residues 1–426, Nt-GFP) still displayed a nuclear localization but failed to associate with chromosomes, suggesting that the C-terminal sequences might be important for targeting BS69 to condensed chromatin (Fig. 3D and data not shown). The above observations prompted us to examine whether BS69 interacts with chromatin-associated factors and to evaluate the contribution of the C terminal. We first examined the ability of a FLAG-tagged BS69 C-terminal peptide (BS69Δ431) to immunoprecipitate chromatin remodeling proteins when transiently transfected in HeLa cells. Of the proteins tested, we found that BS69 interacted with the ATP-dependent helicase BRG1, but not with BAF-155 and BAF-170, which are other components of the Swi-Snf complex (20) (supplemental Fig. S1). We then attempted to confirm in transient transfection the BS69-BRG1 interaction and also searched for additional potential cofactors. BS69 was confirmed to specifically immunoprecipitate the BRG1-related protein brahma (21) but also the histone deacethylase HDAC1 (for review, see Ref. 22) and the histone-methyltransferase EZH2 (23, 24) (Fig. 4, A–C). Inversely, in similar condition, BS69 failed to interact with the histone acetyltransferase pCAF/p300 or
The BS69 proteins were examined by Western blotting using the polyclonal N-terminal peptide-EGFP (or insoluble) expression vectors transfected in Q76 fibroblasts as indicated. BS69, HDAC1, or EZF6 proteins were immunoprecipitated using an α-FLAG M2 (α-F), an α-Myc A14 (α-M), or an α-HA (α-H) antibody as indicated. Co-immunoprecipitation (Co-IP) and ectopic expression of the partner tested was examined by Western blotting (W-B). E, the endogenous BS69 interacts with chromatin remodeling factors. 293T nuclear extracts were immunoprecipitated with an α-BS69 antibody or with non-immune IgG (IgG) as a control. Co-immunoprecipitation of BRG1, EZH2, EZF6, and HDAC1 was examined by Western blotting. F, EZH2 and EZF6 directly bind to BS69 C-terminal sequences in vitro. Top, schematic representation of the BS69 peptides used. The position of the C523S mutation is indicated by a star. Bottom, binding of EZH2 and EZF6 to a wild type (G-431m) or to a mutated (mutated C543S, G-431m) BS69 C-terminal peptide expressed as a GST fusion protein or to the GST (G-) moiety as a control. Ig, input 10%. G, EZF6 immunoprecipitates EZH2, HA-EZF6 and/or Myc-EZH expression vectors were transiently transfected in Q76 fibroblasts. EZF6 proteins were immunoprecipitated with an α-HA antibody (α-H). Co-immunoprecipitation (Co-IP) and ectopic expression of EZH2 was examined by Western blotting (W-B) using an α-Myc A14 antibody. H, BS69 and RACK7 proteins share common partners. 293T nuclear extracts were immunoprecipitated with an α-RACK7 antibody (α-R7) or with non-immune IgG (IgG) as a control. Co-immunoprecipitation of BRG1 and EZF6 were examined by Western blotting.
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DISCUSSION

Our re-examination of BS69 expression led to a number of important adjustments. We corrected the 5′-BS69 cDNA sequence and showed that BS69 is in fact a ubiquitous protein of 74 kDa (Fig. 1). The additional sequences identified are extremely well conserved during evolution, suggesting that their presence could have a significant impact on the analysis of BS69 functions. Importantly, despite the observed diversity in BS69 gene expression, with the characterisation of four splice variants, we failed to detect any BRAM1 encoding cDNA. BRAM1 was reported to consist of five specific residues preceding the C-terminal third of the BS69 protein due to the usage of an internal ATG located in an alternatively spliced exon (18). However, we determined that the proposed BRAM1-specific sequences perfectly map to the anks1 cDNA and also to the location of the anks1 gene on human chromosome 6 (26).

No corresponding sequences are present on chromosome 10 where the BS69 gene is located. It is therefore reasonable to assume that the BRAM1 cDNA is in fact a chimeric construct between anks1 and BS69 sequences that arose from a recombination event during the cDNA library construction. The functions of the PHD- and MYND-less BS69 splice variants described in this study remain to be defined. Because both structures act as protein-protein interaction surfaces (2, 27), the splice variants may either display specific functions or alternatively represent transdominant negative isoforms of BS69. In particular, with the knowledge that the MYND domain of BS69 is targeted by the two oncoviral proteins E1A and EBNA2, it is tempting to speculate that the expression of the MYND-less variants, or the neutralization of full-length BS69 by oncoviral proteins, may have similar consequences that contribute to some extend to the cell transformation process. Interestingly, a MYND-less splice variant of AML1-MTG8 has been recently described in leukemia, which was proposed to stimulate the oligomerization of the protein and potentially favor the malignant conversion of hematopoietic cells (28). Furthermore, as described below, we have already established that there are important differences in the binding partners for the full-length versus the MYND-less variants of BS69.

As for the shorter variants, the function of full-length BS69 is also poorly understood. Previously data regarding BS69 interacting partners has relied predominately on overexpression of tagged proteins due to the lack of a reliable BS69 antibody. We have succeeded in providing an antibody and specific small interfering RNA that should prove valuable for the analysis of BS69 expression and function. We found that all four BS69 isoforms were nuclear, specifically associated with chromatin, and subject to the ubiquitin-proteasome degradation pathway (Figs. 2 and 3). Although the N-terminal half of the protein consists of a combination of motifs (PHD, bromo, and PWWP domains) generally found in gene regulators, deletion of the C-terminal third of the protein strongly impaired the association of BS69 with mitotic chromosomes, suggesting that these sequences also contribute to the interaction of BS69 with chromatin (Fig. 3). In keeping with this hypothesis, we identified four novel BS69 C terminus interacting partners by co-immunoprecipitation of the endogenous proteins, namely E2F6, BRG1, HDAC1, and EZH2 (Fig. 4F). The integrity of the MYND domain structure was found to be a prerequisite only for EZH2 binding, such that the BS69 MYND-less variants were still capable of binding E2F6, but not EZH2, in GST pull-down assays (Fig. 4F and data not shown). Although the binding of E2F6 and EZH2 to independent C-terminal sequences of BS69 and to each other (Fig. 4F) suggested a potential BS69-E2F6-EZH2 ternary complex, we cannot exclude that the binding of BS69 to both partners occurs in completely different contexts. Purification of the BS69-associated protein complexes should help to clarify this point. Our recent findings that the BS69-related protein RACK7 shares a number of common properties and an overlapping set of binding partners with BS69 (Fig. 4H) further suggests that the novel identified partners might be of importance for the functions of both proteins and contribute to their proposed roles in the regulation of gene repression.

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