Sumoylation regulates lamin A function and is lost in lamin A mutants associated with familial cardiomyopathies

Yu-Qian Zhang and Kevin D. Sarge

Department of Molecular and Cellular Biochemistry, Chandler Medical Center, University of Kentucky, Lexington, KY 40536

Lamin A mutations cause many diseases, including cardiomyopathies and Progeria Syndrome. The covalent attachment of small ubiquitin-like modifier (SUMO) polypeptides regulates the function of many proteins. Until now, no examples of human disease-causing mutations that occur within a sumoylation consensus sequence and alter sumoylation were known. We show that lamin A is sumoylated at lysine 201 and that two lamin A mutants associated with familial dilated cardiomyopathy, E203G and E203K, exhibit decreased sumoylation. E203K occupies the conserved +2 position in the sumoylation consensus ΨKXE. Lamin A mutants E203G, E203K, and K201R all exhibit a similar aberrant subcellular localization and are associated with increased cell death. Fibroblasts from an individual with the E203K lamin A mutation also exhibited decreased lamin A sumoylation and increased cell death. These results suggest that SUMO modification is important for normal lamin A function and implicate an involvement for altered sumoylation in the E203G/E203K lamin A cardiomyopathies.

Introduction

The lamin A protein plays an important role in the structure and function of the nucleus, and mutations in the lamin A gene cause a large number of different human diseases, including cardiomyopathies, muscular dystrophies, and Hutchinson-Gilford Progeria Syndrome (Broers et al., 2006; Capell and Collins, 2006; Mattout et al., 2006; Parnaik and Manju, 2006). Covalent attachment of small ubiquitin-like modifier (SUMO) proteins to lysine residues in target proteins, or sumoylation, is an important regulator of protein functional properties (Hay, 2005; Bossis and Melchior, 2006; Kerscher et al., 2006). SUMO proteins are covalently attached to target lysine residues by the SUMO E2 enzyme ubc9, and these substrate lysines are typically found within the consensus sequence ΨKXE (Ψ represents hydrophobic amino acids; Desterro et al., 1997; Johnson and Blobel, 1997; Rodriguez et al., 2001; Sampson et al., 2001). Cells express three major SUMO paralogues, SUMO-1, SUMO-2, and SUMO-3, with SUMO-2 and -3 being much more similar to each other than to SUMO-1 (Hay, 2005; Kerscher et al., 2006; Bossis and Melchior, 2006).

Using a yeast two-hybrid screen, a previous study identified an interaction between lamin A and ubc9, the SUMO E2 protein (Zhong et al., 2005). Based on this interaction, we hypothesized that the lamin A protein could be a target of sumoylation. The purpose of the experiments in this present study was to determine whether lamin A is indeed sumoylated in cells and, if so, what role this modification plays in regulating the function of this lamin.

Results and discussion

First, we sought to test for sumoylation of endogenous lamin A by performing immunoprecipitation of HeLa cell extracts using lamin A antibodies, followed by Western using antibodies against SUMO-1 or SUMO-2/SUMO-3 (because of the similarity of SUMO-2 and -3, it is likely that both of these SUMO proteins are recognized by this antibody). The results suggest that lamin A is SUMO modified and that it is preferentially modified by SUMO-2 compared with SUMO-1 (Fig. 1 A). The results in Fig. 1 C indicate that lamin A protein in extracts of mouse heart is also sumoylated and that, like lamin A that is present in HeLa cell extracts, SUMO-2 appears to be the predominant SUMO protein attached to this protein.

Analysis of the lamin A amino acid sequence revealed a match to the sumoylation consensus sequence ΨKXE (MKEE) surrounding lysine 201 in the rod-containing domain of lamin A (Fig. 2 A, top). To test whether sumoylation of the lamin A is

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occurring at lysine 201, HeLa cells were transfected with mammalian expression plasmids encoding GFP fusion constructs of wild-type lamin A and lamin A in which this lysine was changed to a nonsumoylatable arginine (K201R), along with expression constructs encoding HA-tagged SUMO-1 or -2. Extracts of the transfected cells were subjected to immunoprecipitation with anti-GFP antibodies followed by anti-HA Western blot. The results of this experiment, in agreement with the results obtained using anti–SUMO-2 or –SUMO-1 antibodies. The retransfected cells were subjected to immunoprecipitation with constructs encoding HA-tagged SUMO-1 or -2. Extracts of the cell lysates used for the immunoprecipitations were subjected to immunoprecipitation using anti–SUMO-2 or –SUMO-1 antibodies. (C) Extracts prepared from mouse heart were subjected to immunoprecipitation using anti-lamin A antibodies followed by Western blot assays using antibodies against SUMO-2, SUMO-1, or lamin A (different from those used for immunoprecipitation).
In light of the defective sumoylation and aberrant localization patterns of the K201R, E203G, and E203K lamin A mutant proteins, we hypothesized that expression of these lamin A mutant proteins could result in decreased cell viability. The results shown in Fig. 3 C indicate that this appears to be the case, as all three of these mutant lamin A proteins are associated with increased cell death.

In the final set of experiments, we examined skin fibroblasts obtained from a patient that is heterozygous for the E203K lamin A mutation, which, as already described, is associated with cardiomyopathy (Jakobs et al., 2001). Immunoprecipitation of lamin A from these cells followed by SUMO-2 Western blot indicates that, as expected, levels of sumoylation of lamin A are reduced in cells from this individual compared with cells of a normal individual (Fig. 4 A). This individual is heterozygous for the E203K lamin A mutation, thus we expect that lamin A sumoylation was decreased but not eliminated. Further, immunofluorescence analysis of lamin A indicated more cells showing abnormal lamin A localization and nuclear morphology for the E203K fibroblasts compared with normal fibroblasts (Fig. 4 B). The percentages of normal and E203K fibroblasts exhibiting abnormal lamin A localization/nuclear morphology are shown in Table S1. The E203K fibroblasts also exhibited increased cell death compared with the control fibroblasts (Fig. 4 C).

The results presented in this paper indicate that lysine 201 of lamin A is a target of covalent modification by the SUMO-2 protein and that this sumoylation is important for the normal pattern of subcellular localization of the lamin A protein. The results of these experiments also show that lamin A sumoylation is decreased in transfected mutant E203G and E203K lamin A proteins that cause familial dilated cardiomyopathies and in lamin A of fibroblasts of individuals with the E203K mutation. To our knowledge these are the first examples of human disease-causing mutations occurring in a crucial residue of a sumoylation consensus sequence and resulting in decreased sumoylation of the mutant protein.

The results also indicate that the mutant E203G and E203K lamin A proteins exhibit altered subcellular localization patterns that are very similar to that of the SUMO attachment site mutant K201R lamin A protein. These results suggest a role for sumoylation in the correct localization of lamin A in the cell. Further, cells transfected with the E203G and E203K lamin A proteins and skin fibroblasts of individuals with the E203K mutation exhibit higher levels of cell death compared with cells transfected with wild-type lamin A or normal skin fibroblasts, respectively. Together, these results suggest that sumoylation is important for normal lamin A function and support a role for altered sumoylation in the underlying molecular mechanism of familial dilated cardiomyopathies associated with the E203G/ E203K lamin A mutations.

Materials and methods

Cell culture and plasmids

HeLa cells were cultured in DME medium (Mediatech, Inc.) supplemented with 10% FBS and 100x antibiotic-antimycotic (Invitrogen) in 5% CO₂. Human skin fibroblasts (provided by R. Hershberger, University of Miami, Miami, FL) were cultured in the same media supplemented with 2 mM glutamine. Transfection of HeLa cells was performed using Effectene transfection reagent (Qiagen), according to the manufacturer’s protocol. Immunoprecipitation analysis and fluorescence microscopy were performed 48 h after the transfection. The GFP-lamin A plasmid was constructed from the pcDNA3.1-LMNA plasmid (gift of N. Zhong and W.T. Brown, Institute for Basic Research in Developmental Disabilities, Staten Island, NY). The coding
The beads were washed with RIPA buffer four times and then boiled in protein G – Sepharose slurry was added and incubated at 4 °C for 60 min. (BD Biosciences). After incubation at 4 °C for 60 min, 150 μl of 50% anti-GFP antibody (Bethyl Laboratories, Inc.) or anti–lamin A antibody at 4 °C for 60 min. After centrifugation at 4,000 rpm at 4 °C for 1 min, mixing it with 150 μl of this slurry and 5 μg of goat IgG and incubating RIPA buffer to make a 50% slurry. The cell lysate was precleared by transfected GFP–lamin A. 300 μl of 50% protein G – Sepharose slurry was added and incubated at 4 °C for 60 min. After centrifugation at 10,000 rpm at 4 °C for 10 min, the supernatant was transferred to a fresh tube, and 20 μl of the whole cell lysate was removed for analysis of the level of transfected GFP–lamin A. 300 μl of 50% protein G–Sepharose slurry (GE Healthcare) was washed three times with PBS and resuspended in RIPA buffer to make a 50% slurry. The cell lysate was preclarified by mixing it with 150 μl of this slurry and 5 μg of goat IgG and incubating at 4 °C for 60 min. After centrifugation at 4,000 rpm at 4 °C for 1 min, the supernatant was transferred to a fresh tube and mixed with 5 μg of anti-GFP antibody (Bethyl Laboratories, Inc.) or anti–lamin A antibody (BD Biosciences). After incubation at 4 °C for 60 min, 150 μl of 50% protein G–Sepharose slurry was added and incubated at 4 °C for 60 min. The beads were washed with RIPA buffer four times and then boiled in 50 μl of 4 × SDS-PAGE loading buffer. After brief centrifugation, the supernatants were subjected to SDS-PAGE and Western blot using the antibodies described in the next section.

Western blot analysis and antibodies
SDS-PAGE and Western blot were performed according to standard procedures. The antibodies and dilutions used to probe the Western blots were as follows. Goat anti-GFP antibody (Bethyl Laboratories, Inc.) was used at 1:2,000, mouse anti-HA antibody (gift from D. Andrews laboratory, University of Kentucky, Lexington, KY) was used at 1:2,000, goat anti–SUMO-1 antibody (Bethyl Laboratories, Inc.) was used at 1:1,000, rabbit anti–SUMO-2 antibody (Abgent) was used at 1:500, and rabbit anti–lamin A antibody (Abcam) was used at 1:500.

Statistical analysis
Statistical significance was determined using Student’s t test. A p-value of <0.05 was considered to be statistically significant.
Online supplemental material

Table S1 contains quantitation analysis for the experiments in Fig. 3 (A and B) and Fig. 4 B. This table shows the percentages of HeLa cells or cardiomyocytes transfected with GFP lamin A wildtype, K201R, E203G, or E203K mutants that exhibit abnormal lamin A localization and the percentages of normal versus E203K lamin A fibroblasts showing abnormal lamin A localization/nuclear morphology. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712124/DC1.

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Preparation of cardiomyocytes

These cells were provided by W. Lester and J. Satin [University of Kentucky, Lexington, KY]. All animals and animal procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. E16 mouse (ICR outbred strain; Harlan) hearts were dissected free of connective tissues, and ventricles were separated from conotruncus and sinus venosus or atria. Cells were enzymatically dispersed and cultured as previously described (Cribbs et al., 2001). In brief, 10–40 embryos were minced and quickly transferred to nominally Ca2+-free digestion buffer containing 0.5 mg/ml collagenase (type II; Worthington) and 1 mg/ml pancreatin for two 15-min cycles. Digested tissue yielded a large fraction of single spontaneously beating cells in culture media consisting of DME containing 10% FBS.

Figure 4.

Fibroblasts from an individual with E203K lamin A mutation exhibit decreased lamin A sumoylation, altered lamin A localization/nuclear morphology, and increased cell death. (A) Extracts prepared from skin fibroblasts of a normal individual and the individual heterozygous for the E203K lamin A mutation were subjected to immunoprecipitation using anti–lamin A antibodies. DNA was immunoprecipitated. (B) Skin fibroblasts of a normal individual and the individual heterozygous for the E203K lamin A mutation were subjected to immunoprecipitation using anti–lamin A antibodies (different from those used for immunoprecipitation). (B) Skin fibroblasts of a normal individual and individual heterozygous for the E203K lamin A mutation were subjected to immunofluorescence analysis using anti–lamin A antibodies. DNA was visualized by staining with HOECHST 33342. Bar, 5 μm. (C) Skin fibroblasts of a normal individual and E203K lamin A individual were analyzed by trypan blue assay to measure cell death. Data are shown as means ± SEM (P < 0.0038 for E203K lamin A mutant cells vs. wild-type cells) and are from three datasets.