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Biochemical and Biophysical Research Communications 345 (2006) 74–77
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www.elsevier.com/locate/ybbrc

Modification of Msx1 by SUMO-1

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Received 24 March 2006
Available online 19 April 2006

Abstract

The small ubiquitin-related modifier SUMO reversibly modifies many proteins, including promoter-specific transcription factors. Genetic studies in both humans and mice indicate that the Msx1 transcription factor is associated with specific disorders, including cleft palate. We show that Msx1 conjugation to SUMO-1 in vivo is enhanced by an E3 SUMO ligase, PIAS1, suggesting that sumoylation of Msx1 is a new mechanism for modulating the molecular function of Msx1 during organogenesis.

Keywords: Msx1; Sumoylation; Post-translational modifications

Post-translational modification of proteins by the small ubiquitin-related modifier (SUMO) is increasingly recognized as an important regulatory mechanism. The conjugation of SUMO to target proteins (SUMOylation) leads to the formation of an isopeptide bond between the C-terminus of SUMO and an ε-amino group of a lysine residue in the target protein [1]. In most cases, the lysine residue is embedded in a consensus sequence composed of a characteristic ΨKXE motif, where Ψ is a large hydrophobic amino acid, X is any residue, and K (lysine) is the site of SUMO-conjugation [1–3]. The SUMO pathway mechanistically resembles that of ubiquitination, but the enzymes involved in the two processes are distinct. SUMOylation utilizes the heterodimeric E1 SUMO-activating enzyme SAE1/SAE2, the E2 SUMO conjugating enzyme Ubc9, and recently identified E3 ligases for substrate selection and reaction specificity [4–6]. Among the ligases is the family of PIAS (protein inhibitors of activated STATs) nuclear proteins that function as SUMO ligases for STAT and for other proteins [6]. As a dynamic process, SUMOylation is readily reversed by a family of SUMO specific proteases (SENP family members in mammals), thus providing a control mechanism for the modified protein by the cell [7].

The proteins targeted by SUMO fall into different categories such as signal transducers, enzymes, viral proteins, regulators of chromatin structure, DNA repair proteins, and transcription factors [1,2]. Sumoylation of transcription factors has been shown to affect their stability, localization, and activity as activators or repressors, by altering protein–protein interactions to favor recruitment of co-repressors, by regulating their sub-nuclear localization, by inducing conformational changes in the structure of the transcription factor or by competing with other post-translational modifications for lysine residues [8–11]. Msx1 is a member of the Msx transcription factors that play key roles in development by repressing gene expression through interactions with components of the core transcription complex as well as with other homeoprotein [12,13]. Mutations in both human and mice indicate that Msx1 is associated with specific human craniofacial disorders including cleft palate and anodontia [12]. Here, we show that Msx1 is sumoylated in vivo and we propose that Msx1 sumoylation constitutes a new regulatory mechanism modulating Msx1 function during organogenesis.

Materials and methods

Computational studies. Putative sumoylation sites in Msx1 (NCBI Accession No: BC016426) were identified using SUMOPLOT (Abgent, Inc., CA). Multiple sequence clustering for phylogenetic analysis was

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doi:10.1016/j.bbrc.2006.03.232

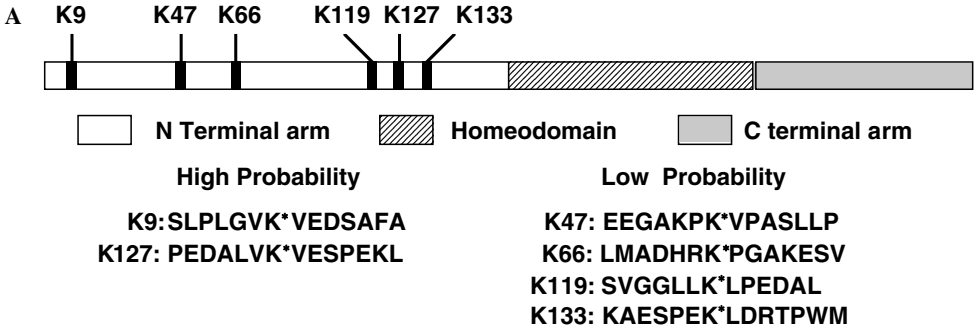
carried out with ClustalW. The sequences used for the phylogenetic analysis were *Danio rerio* (NM_131273.1), *Xenopus laevis* (BC081101), *Gallus gallus* (BAA01209.1), *Mus musculus* (BC016426), and *Homo sapiens* (AAA52683).

Antibodies. Mouse monoclonal anti-FLAG antibody M2 and mouse monoclonal anti-HA, mouse monoclonal anti-Msx1 and mouse monoclonal anti-GMP-1 (SUMO-1) were purchased from Sigma, Covance and Zymed, respectively. Horseradish peroxidase-conjugated anti-mouse IgG was purchased from Amersham Biosciences.

Expression cloning. The plasmid pCMV-FLAG-Msx1 encoding wild-type full-length Msx1 tagged with FLAG epitope was constructed as follows: The Msx1 ORF was amplified by PCR using 5′-CGCGGATCCA TGACTTCTTTGCCACTCGG-3′ as the forward primer and 5′-CCAA GCTTCTAAGTCAGGTGGTACATGCTGTAT as the reverse primer. The PCR product was digested with *Bam*HI and *Hin*dIII, and cloned in the corresponding *Bam*HI- and *Hin*dIII-restricted pCMV-FLAG plasmid (Stratagene, La Jolla, CA). Point mutations K9R, K47R, K66R, K119R, K127R, and K133R in pCMV-Msx1-Flag were generated by PCR amplification using mutated oligonucleotides employing GeneTailor Site-Directed Mutagenesis system (Invitrogen, Carlsbad, CA). The following oligonucleotide primers were used: sense K9R: 5′-CACTCGGTGTCA GAGTGGAGGACTC-3′; antisense K9R: 5′-GAGTCCTCCACTCTGA CACCGAGTG-3′; sense K47R: 5′-AGGGGGCCAAGCCCAGAGTG CCCGCT-3′; antisense K47R: 5′-GTCTACTCCTCCCCCGGTTCGGG T-3′; sense K66R: 5′-CCGATCAC AGGAGGCCCGGGGCCA AG-3′; antisense K66R 5′-CTTGGCCCCGGGCCTCCTGTGATCGG-3′; sense K119R: 5′-GGAGGACTCCTCAGGCTGCCAGAAGATG-3′; antisense K119R: 5′-CATCTTCTGGCAGCCTGAGGAGTCCTCC-3′; sense K127R: 5′-GATGCTCTGGTGAGGGCCGAAAGCCC-3′; antisense 5′-GGGCTTTCGGCCCTCACCAGAGCATC-3′; sense K133R: 5′-CCGA AAGCCCCGAGAGACTAGATCGGAC-3′; antisense K133R: 5′-GAC CACTTCCGGCTTTCGGGGCTCT-3′. Wild-type SUMO-1-GFP and mutant SUMO1-GFP were kindly provided, by Dr. Palvimo (University of Helsinki). PIAS1 was provided by Dr. Shuai (University of California, LA). HA-tagged SUMO-2 and SUMO-3 were provided by Dr. R. Hay (University of St. Andrews, Scotland) and Senp1 was obtained from Dr. E. Yeh (The University of Texas-M.D. Anderson Cancer Center).

Cell culture and transfections. C3H10T1/2 cells were obtained from American Type Culture Collection and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 10 U/ml penicillin, 10 μg/ml streptomycin, and 25 ng/ml amphotericin B (Invitrogen). 3×10^5 cells/well were plated in 6-well plates and next day transient transfections were performed with Lipofectamine (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Each transfection was repeated at least three times.

Preparation of cell lysates and Western blotting. Cells were harvested after 36 h and lysed in a modified RIPA buffer containing 50 mM Tris (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonident P-40, and 0.1% sodium deoxycholate with complete protease inhibitors (Roche Molecular Biochemicals), and 10 mM *N*-ethylmaleimide (NEM). One hundred micrograms of protein was loaded (per lane) for Western blotting experiments. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane. Blots were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dried milk for 2 h at room temperature. Primary antibodies were incubated overnight at 4 °C and washed three times each in TBST. Horseradish peroxidase-conjugated IgG secondary antibodies (Amersham Lifesciences) were added to TBST and membranes were incubated for 1 h in secondary antibody followed by three washes in TBST (10 min each). Western blots were incubated with ECL Western blotting reagent (Amersham Biosciences), exposed to X-ray film, and developed.



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Danio rerio      ----------MAPVVTMSSQAAEED----AGVMLAGQ---------TEMQTRVTVQEDAQ 37
Xenopus laevis   SQLGSLCMAPALLMASYQPGVKIEE---RPLNRMQQTG----------VKPSLGED---- 57
Gallus gallus    -------MAPAADMTTAPTGVRSDEPPASAFSKPGGG---------LPVAAAMGGEEESD 44
Bos taurus       -------------MTSLPLGVKVED---PPFGKPAGGGGGQTPSTTAATAAAMGADAEGA 44
Mus musculus     -------------MTSLPLGVKVED---SAFAKPAGGGVGQAPGAAAATATAMGTDEEGA 44
Homo sapiens     EGGARLCMAPAADMTSLPLGVKVED---SAFGKPAGGGAGQAPSAAAATAAAMGADEEGA 117

Danio rerio      EDAQRPKILPFSVEALMAD-RRPNRGS---------------------------------- 59
Xenopus laevis   KPKVP-GILPFSVEALMAD-RKPG-RDRDLSSPTGSPLAGTSHSPR---VGSIAAG--ET 109
Gallus gallus    KPKVSPSPLPFSVEALMADRRKP---------PGGRDGPEGSGPPLGSARANLGALTTEA 95
Bos taurus       KPKVSPSLLPFSVEALMADHRKPGAKKSVLAASEGAQAAGGSAKPLGARPGSLAAP--DA 102
Mus musculus     KPKVPASLLPFSVEALMADHRKPGAKESVLVASEGAQAAGGSVQHLGTRPGSLGAP--DA 102
Homo sapiens     KPKVSPSLLPFSVEALMADHRKPGAKESALAPSEGVQAAGGSAQPLGVPPGSLGAP--DA 175

Danio rerio      PDADARLG----FSVEVLQLP----VKAESPE---RSTWVPRARFSPS---RLSPPACPL 105
Xenopus laevis   PNSPISLGNRY-PVGAIMQLPEETLLKPESPE---RSSWIQSPSFSPSPTRRMSPPACPL 165
Gallus gallus    PTSPLPLGGHFPSVGALGKLPEDALLKAESPEKPERSPWMQSPRFSPPPPRRLSPPACTL 155
Bos taurus       PSSPRPLG-HF-SVGGLLKLPEDALVKAESPEKPERTPWMQNPRFSPPPSRRLSPPACTL 160
Mus musculus     PSSPRPLG-HF-SVGGLLKLPEDALVKAESPEKLDRTPWMQSPRFSPPPARRLSPPACTL 160
Homo sapiens     PSSPRPLG-HF-SVGGLLKLPEDALVKAESPEKPERTPWMQSPRFSPPPARRLSPPACTL 233
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Fig. 1. Msx1 sumoylation sites. (A) Schematic representation of putative sumoylation sites in Msx1. (B) Phylogenetic sequence comparison of the sumoylation sites (in box) of Msx1. Asterisks represent the position of lysines in murine Msx1.