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Interaction of Daxx, a Fas Binding Protein, with Sentrin and Ubc9

Seung-Wook Ryu, Suhn-Kee Chae, and Eunhee Kim[1]
Research Center for Biomedicinal Resources and Division of Life Science, PaiChai University, 439-6 Doma-dong, Seo-gu, Taejon 302-735, South Korea

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Sentrin is a ubiquitin-like protein that can covalently modify cellular proteins, and is a Fas binding protein that protects cells against anti-Fas induced cell death. However, the mechanism by which sentrin exerts its effect upon Fas-mediated apoptosis is not well known. Thus, this study examined the interaction of sentrin with Daxx. Sentrin interacted with Daxx but not with FADD when analyzed by yeast two-hybrid assay. *In vitro* translated Daxx bound to GST-sentrin fusion protein. FLAG-sentrin fusion protein was also coimmunoprecipitated with Daxx in BOSC23 cells. Also, Daxx interacted with Ubc9, an essential protein as a key conjugating enzyme. Amino acids 625–740 of Daxx, known as Fas binding region, was also mapped as sentrin and Ubc9 binding region. Colocalization of Fas, sentrin, and Ubc9 binding regions suggests the importance of that region upon the regulation of Daxx. Our data also demonstrated that sentrin could homo-oligomerize by protein–protein interaction. © 2000 Academic Press

Key Words: **Daxx; sentrin; Ubc9; sentrinization; domain.**

Sentrin was originally isolated by the yeast two-hybrid system with the death domain of Fas as a bait (1). Sentrin is a ubiquitin-related protein and has many different names PIC1, GMP1, UBL1, SMT3C, and SUMO-1, respectively (2–6). Sentrin antibody stained an 18 kDa sentrin monomer, a 90 kDa band, and multiple high molecular weight bands (7). Sentrin has ubiquitin-like domain (a.a. 22–97). However, sentrin contains four additional amino acids (His-Ser-Thr-Val) at the C-terminus, which are cleaved so as to allow the conjugation of sentrin to other proteins via the glycine residue at the C-terminus (7).

Protein modification by sentrin is clearly distinct from ubiquitination. Conjugation of ubiquitin proceeds via a reaction cascade involving ubiquitin-activation (E1), ubiquitin-conjugating (E2) enzymes, and sometimes ubiquitin protein ligases (E3) (8–10). However, modification by sentrin requires initial activation of the Gly-97 by a ubiquitin-conjugating enzyme, Ubc9 (11–13). The PML, RanGAP1, and Werner's syndrome gene product have been recently reported as the targets for sentrinization with Ubc9 (12–14). Ubiquitination is essential for the proteins to be degraded substrate by proteasomes (15–17). Unlike ubiquitin modification, sentrinization is clearly not a signal for degradation. The roles of sentrinization (or sumoylation) have been suggested as nuclear translocation of target proteins and obstruction of cell death signaling by prevention of FADD or TRADD binding to Fas and TNFR1 (1, 3).

Fas-induced apoptosis has been reported to have two distinct pathways mediated by adapter proteins FADD recruiting pro-caspase-8 and Daxx activating the ASK1 (apoptosis signal regulating kinase) (18). Daxx was identified as a Fas binding protein implicating in the enhancement of Fas-mediated apoptosis and the activation of Jun N-terminal kinase (18–20). Daxx encodes a protein of 740 amino acids and migrates a molecular weight of approximately 110, 97, and 70 kDa as results by post-translational hyperphosporylation (21–22). Daxx has been observed in the nuclei and to a lesser extent in the cytoplasm in COS-1 cell line. Daxx repressed the transcriptional activity of Pax3, a member of the paired class homeodomain family transcription factor (22). Also, Daxx interacted with modified PML by sentrin and served as a component of nuclear domain 10 (ND10), also referred to as PML bodies or PODs (23–25). These results implicate putative nuclear functions of Daxx. However Daxx is obviously too big to passively diffuse into the nucleus. The nuclear translocation mechanism of Daxx is largely unknown.

The physiological role of sentrin-Fas binding is not well understood except the fact that sentrin protects cells against anti-Fas induced cell death. The mecha-

[1] To whom correspondence should be addressed. Fax: 82-42-520-5463. E-mail: ehkim@mail.paichai.ac.kr.

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nism by which sentrin exerts its effect upon Fas signaling has not been studied carefully. This study aimed to examine the interaction between sentrin and Daxx, another signal transducer of Fas.

MATERIALS AND METHODS

Materials, media, strain, and plasmids. All chemicals used for yeast transformation and β-galactosidase assays were purchased from SIGMA (USA). Restriction enzymes and ligases were obtained from GIBCOBRL (USA) and *Taq* DNA polymerase was purchased from TaKaRa (Japan). Synthetic dropout minimal (SD) medium with various supplements were used for yeast culture, maintenance, and selection of transformants. Yeast strains, EGY48 (*Matα, ura3-52, his3, trp1, leu2::pLeu2-LexAop6/pSH18-34[LexAop-lacZ]*) were purchased from Clontech Laboratories, Inc. (USA). pLexA vector containing *E. coli* LexA DNA binding domain (DBD) and pJG4-5 vector containing *E. coli* B42 activation domain (AD) sequences were purchased from Clontech Laboratories, Inc. (USA) as parts of the MatchMaker two-hybrid assay kits.

Construction of vectors expressing fusion proteins in yeast two-hybrid system. In order to clone sentrin, Daxx full length size, 330 to 740 amino acids of Daxx (Daxx 330–740), 625 to 740 amino acids of Daxx (Daxx 625–740), FADD, TRADD, and Ubc9, PCRs were performed using HeLa cDNA library as template using following primers.

Sentrin primers (5′-CACGGATCCCATGTCTGACCAGGAG-3′ and 5′-CCCCTCGAGCTA AACTGTTGAATG-3′), Daxx full length primers (5′-TCAAGAATTCATGGCCACCGCTAACA GCATC-3′ and 5′-TTGCTCGAGCTAATCAGAGTCTGAGAGCACGATG-3′), Daxx 330–740 primers (5′-CGAGAATTCCTCGATCTCATCTACAAC-3′ and 5′-TTGCTCGAGCTAATCAGAG TCTGAGAGCACGATG-3′), Daxx 625–740 primers (5′-TCGAATTCTCTGGTCCCCCCTGCA AAAAATC-3′ and 5′-TTGCTCGAGCTAATCAGAGTC-TGAGAGCACGATG-3′), FADD primers (5′-ATGAATTC-ATGGACCCGTTCCTGGTGCTGCTGC-3′ and 5′-ATACT-CGAGTCAGGACG CTTCGGAGGTAGATGCG-3′), TRADD primers (5′-AGGAGAATTCATGGCAGCTGGGCAAA ATG-3′ and 5′-CACGTCGACTCTAGGCCAGGCCGCCAT-3′), and Ubc9 primers (5′-CCTGAA TTCATGTCGGGGATCGCCCTC-3′ and 5′-CCACTCGAGCGCTGCTTATGAGGGCGC-3′) were designed from the sequence information by GenBank database.

PCR products of sentrin (306 bp), FADD (646 bp), and Ubc9 (500 bp) were fused in-frame with LexA DBD. Daxx full length size (2,223 bp), Daxx 330–740 (1,230 bp), Daxx 625–740 (348 bp), sentrin, TRADD (986 bp), and FADD were fused in-frame with B42 AD.

Yeast transformation and β-galactosidase assay. Yeast strain EGY48 was co-transformed with a possible pair of pLexA and pJG4-5 constructs and analyzed for the interactions. The β-galactosidase activity of an *in vivo* plate assay and of liquid cultures using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate were performed by the method described from Clontech Laboratories, Inc. (USA). Briefly, in the plate β-galactosidase assay for blue/white detection directly on SD medium lacking uracil (Ura), histidine (His), and tryptophan (Trp) for pLexA and pJG4-5 derived constructs, 5-bromo-4chloro-3-indolyl-D-galactopyranoside (X-gal) was added at a concentration of 40 μg/ml to melted SD medium. Blue colors on the plates containing X-gal were analyzed after 2–3 days. The cultured transformants were monitored by the absorbance at 600 nm until the cells are in mid-log phase. The culture broth was harvested and mixed with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgSO_4$, pH 7.0). Cell mixture containing Z buffer was frozen in liquid nitrogen and then, thawed at 37°C. Z buffer containing β-mercaptoethanol and ONPG were added in thawed cell mixture, and the reaction was carried out at 30°C until yellow color was obtained. The reaction was stopped by the addition of 300 mM Na_2CO_3. Cells were removed by centrifugation, and the absorbance of the supernatant was measured at 420 nm.

GST pull-down assay. Sentrin was obtained from pLexA/sentrin with *Bam*H1 and *Xho*1 digestions. Sentrin was subcloned into the *Bam*H1/*Xho*1 site of pGEX5T-1 (Amersham Pharmacia Biotech, UK). GST-fusion proteins were expressed in *E. coli* BL21(DE3) with IPTG (isopropyl-B-D-thiogalactopyranoside) induction. Subsequently cells were sonicated in ice-cold lysis buffer (200 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 100 μM EDTA, 0.1% Triton X-100, 0.4 mM PMSF). Recombinant GST fusion protein was incubated with glutathione coated beads (Amersham Pharmacia Biotech, UK) for 16 h at 4°C and normalized for protein concentration.

Daxx insert was obtained from pJG4-5/Daxx with *Eco*R1 and *Xho*1 digestions. Daxx was subcloned into pcDNA3 (INVITROGEN, USA). pcDNA3/Daxx was translated *in vitro* with the T_NT transcription/translation system kit (Promega, USA). Briefly, 2 μg of pcDNA3/Daxx DNA was incubated with 20 μCi of [^{35}S]-methionine in T_NT quick master mix for 90 min at 30°C. Equal amounts of aliquots were mixed with either GST-sentrin fusion or GST protein, respectively. *In vitro* translated Daxx was mixed with GST-sentrin fusion bound onto glutathione coated beads in the binding buffer (50 mM Hepes, pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1% NP40, 10% glycerol) and incubated for 16 h at 4°C. After washing three times in lysis buffer, samples were treated with SDS-loading buffer containing 5% β-mercaptoethanol. The samples were loaded to SDS–PAGE and visualized by autoradiography.

Coimmunoprecipitation. The BOSC23 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 100 μg/ml streptomycin (GIBCO BRL, USA). For coimmunoprecipitation, 1×10^6 cells were plated in 10 mm dishes and transfected the following day with mammalian expression vectors encoding the FLAG epitope tagged sentrin (pFLAG-CMV-2/sentrin) by the calcium phosphate precipitation method (26). After 48 h cells were harvested and lyzed in lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.4 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The cell debris was removed by centrifugation at 10,000*g* for 15 min at 4°C. The resulting supernatant was incubated with mouse monoclonal anti-FLAG M2 affinity gel (SIGMA, USA) overnight at 4°C on a rotary shaker. Subsequently, immune complexes were washed with above-mentioned lysis buffer. The immune complexes were separated by 10% SDS–PAGE and transferred to nitrocellulose. Western blot was analyzed by rabbit anti-Daxx antibody (Santa Cruz Biotechnology, USA).

RESULTS

Two-hybrid analysis of sentrin and downstream molecules of Fas. In order to test whether sentrin interacts with members of Fas signaling using the yeast two-hybrid system, sentrin was fused in-frame with LexA DNA binding domain. Fas, FADD, and Daxx were fused in-frame with B42 transcription activation domain. Cotransformants were streaked on the selection media plate (Ura^-, His^-, Trp^-) containing 2% galactose and X-gal for *in vivo* plate assay, and also assayed for the β-galactosidase enzyme activity using ONPG as a substrate (Fig. 1). Cotransformation of pLexA/sentrin and pJG4-5/Daxx resulted in the expression of the reporters *LacZ*. Moreover sentrin did not bind with the FADD and Fas^{lpr}, a Fas mutant as shown in Okura *et al.* (1).

We also tested whether sentrin could form homo-oligomers by protein-protein interaction. As shown in Fig. 1, pLexA/sentrin and pJG4-5/sentrin interacted in

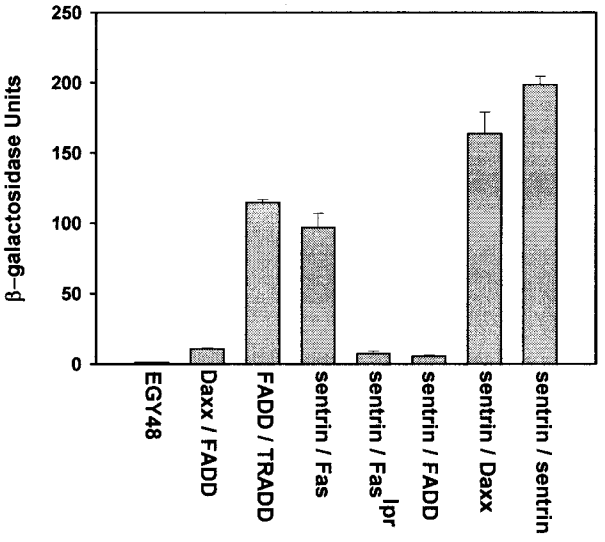

FIG. 1. Interaction analysis of sentrin with Fas signal transducers in yeast. The liquid β-galactosidase assays were carried out in the transformants of yeast strain EGY48 that cotransformed by pLexA/sentrin and FADD, Fas, Fas^{lpr}, Daxx, sentrin in pJG4-5. One unit of β-galactosidase activity was defined as the amount of enzyme required to hydrolyze 1 μmole of ONPG to o-nitrophenol and D-galactose per min. The average values with standard errors of β-galactosidase units were indicated based on the four sets of independent experiments. Each set assaying β-galactosidase activities of the five kinds of transformants plus a positive control (FADD/TRADD) and negative control (EGY48, Daxx/FADD) were carried out at the same time.

yeast two-hybrid system. Kamitani *et al.* (12) reported that sentrin did not form a branched poly-sentrin chain because sentrin lacked the conserved Lys-48 equivalent required for multimer formation. However, our data demonstrated sentrin could homo-oligomerize by protein-protein interaction.

Sentrin interacts with Daxx in vitro and in mammalian cells. In order to examine whether sentrin is able to interact with Daxx *in vitro,* glutathion-S-transferase (GST) and GST-sentrin fusion protein were expressed in *E. coli* strain BL21(DE3) using pGEX5T-1 and pGEX5T-1/sentrin, respectively. As shown in Fig. 2, *in vitro* translated Daxx migrated with molecular weight of approximately a single 110 kDa as ^{35}S-labeled protein on 10% SDS–PAGE. Our data agrees with the description of Yang *et al.* (19) and Hollenbach *et al.* (22) that approximately 110 kDa protein is present because of the presence of abundant acidic residues and hyperphosphorylation in Daxx. Recombinant GST-sentrin and GST only appeared as 45 and 26 kDa protein, respectively. *In vitro* translated Daxx was only retained on matrices to which GST-sentrin had bound, but not GST only (Fig. 2). Thus the interaction of sentrin and Daxx was demonstrated in yeast two-hybrid assay and *in vitro* protein binding assay.

To show that the interaction between sentrin and Daxx can occur in mammalian cells, pFLAG-CMV-2/

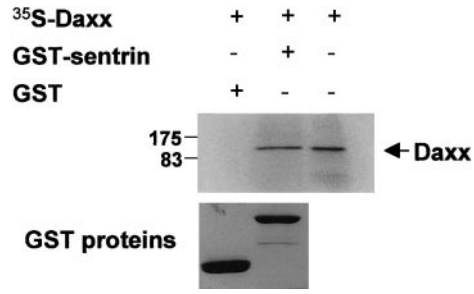

FIG. 2. Interaction of GST-sentrin with *in vitro* translated Daxx. Input of ^{35}S-Daxx protein in binding assays are shown in lane 3. Reaction products were resolved on a 10% SDS–polyacryl-amide gel. Numbers at left indicate protein molecular weight standards in kDa. Coomassie stained GST fusion proteins from the same gel were aligned to show protein levels (bottom panel).

sentrin was transiently transfected in BOSC23 cells. As shown in Fig. 3, Daxx was detected as hyperphosphorylated 110 kDa band by Western blot analysis with rabbit anti-Daxx antibody after immunoprecipitation with anti-FLAG antibody in pFLAG-CMV-2/sentrin transfected BOSC23 cells. But, Daxx was not detected in BOSC23 cells with transfection of pFLAG-CMV-2. These results confirmed the interaction between sentrin and Daxx *in vivo.*

Determination of sentrin and Ubc9 interacting region of Daxx. To map the interacting region between sentrin and Daxx, deletion mutants of Daxx were constructed by PCR, such as pJG4-5/Daxx 330–740 and pJG4-5/Daxx 625–740. Their interaction activities were compared by the expression of the *LacZ* reporter gene by two-hybrid assay in EGY48. Among various deletion constructs, the construct with 625–740 amino acid of Daxx was sufficient to bind with sentrin (Fig. 4). Daxx 625–740 was previously mapped as the binding site of Fas (19).

Also, in order to map interacting region among Daxx, sentrin and Ubc9 by two-hybrid assay, deletion mutants of Daxx were transformed with pLexA/Ubc9 in EGY48. As shown in Fig. 4, pJG4-5/Daxx also interacted with the pLexA/Ubc9. And pJG4-5/Daxx 625–740 capable of sentrin binding was mapped as Ubc9 interacting region.

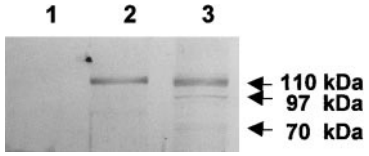

FIG. 3. Coimmunoprecipitation of sentrin and Daxx in BOSC23 cells. The BOSC23 cells were transfected with pFLAG-CMV-2 (lane 1) or pFLAG-CMV-2/sentrin (lane 2). Total cell lysates were loaded in lane 3. Sentrin was immunoprecipitated (lane 1 and lane 2) with mouse anti-Flag and Western blot was carried out with rabbit anti-Daxx antibody. Arrows indicate Daxx proteins as molecular weight of 70, 97, and 110 kDa.

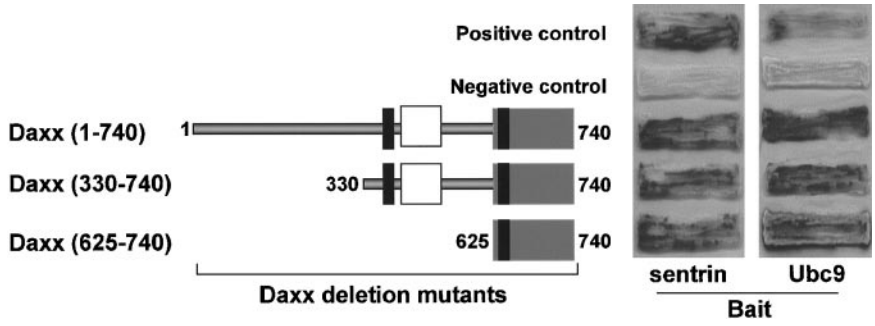

FIG. 4. Interacting region of Daxx with sentrin and Ubc9. The yeast two-hybrid system was used to map the interacting sites of Daxx deletion mutants with sentrin and Ubc9, respectively. The wild type and the deletion mutants with indicated sequences (amino acids in parenthesis) of Daxx were fused to the B42 activation domain. The sentrin and Ubc9 were fused LexA DNA-binding domain as bait. Black box, blank box, and shaded box indicate the nuclear localization signal (NLS), acidic amino acid rich region, and Fas binding region, respectively. The cotransformants of EGY48 with pJG4-5/TRADD and pLexA/FADD as positive controls and those of pJG4-5/Daxx and pLexA/FADD as negative controls were streaked on SD medium containing X-gal as substrate for β-galactosidase.

DISCUSSION

In this study, we observed that sentrin interacts with Daxx. And the sentrin binding domain in Daxx colocalizes with that of Fas binding. Amino acid 625–739 region of mouse Daxx was mapped as Fas interacting domain (19). This domain contains several Ser/Pro/Thr-motifs which are commonly found in transcriptional regulators. This region is also known to be hyperposphorylated (22). This study also mapped amino acid 625–740 region of human Daxx as sentrin and Ubc9 binding region. Thus this region seems to be important in the physiological regulation of Daxx. Our data suggest that Daxx may be functionally regulated by sentrinization.

Conjugation of sentrin to its target proteins by Ubc9 occurs between glycine residue in C-terminal of sentrin and lysine residue in a target protein (11, 12, 27). Eight lysine residues exist in amino acid 625–740 region of human Daxx which was mapped as sentrin and Ubc9 binding region. Therefore, it is likely that one of eight lysine residues may be covalently modified by sentrin with Ubc9 if Daxx is sentrinized.

In this study, the radiolabeled product of 110 kDa appeared predominantly in *in vitro* transcription/translation system with human Daxx cDNA. According to the studies of Hollenbach *et al.* (22), Daxx migrated with apparent molecular weights of 70, 97, and 110 kDa by the degree of phosphorylation and 70 kDa is the nonphosphorylated form of Daxx. Therefore, 110 kDa of *in vitro* translated Daxx (Fig. 2) in our data seem to be the result by phosphorylation in *in vitro* transcription/translation system with rabbit reticulocyte lysate. Endogenous Daxx was detected predominantly as 110 kDa and to a much lesser extent as 97 and 70 kDa protein in BOSC23 cells (Fig. 3). However only 110 kDa of Daxx protein was immunoprecipitated with FLAG-sentrin. Thus, our data show that phosphorylated Daxx interacts with sentrin. However it is hard to judge if 97 and 70 kDa forms of Daxx could interact with sentrin because of their rare presence.

Since Daxx was discovered as Fas binding protein, Daxx has been expected to be present in the cytoplasm (19). However Daxx was observed predominantly in the nucleus of Daxx transfected COS-1 cell (21). Two nuclear translocation signals exist within Daxx. However experiments were not done if those signals are functionally sufficient. The nuclear translocation mechanism of Daxx is not yet known. The Daxx/sentrin and Daxx/Ubc9 interaction from this study and nuclear presence of Daxx leads us to ask the question if Daxx is sentrinized. Sentrinization of Daxx is currently under study.

The fact that sentrin attenuates Fas signaling and binds to Daxx leads us to contemplate that sentrin deviates Daxx from Fas signaling. Moreover if Daxx is sentrinized, sentrin might serve as nuclear translocation vehicle of Daxx. Thus Daxx might leave from Fas modulating and switch to its nuclear function. Even if Daxx is not sentrinized, sentrin still can influence Fas signaling by competing with Fas for Daxx binding. Thus it is necessary to delineate the characteristics of sentrin/Daxx and Fas/Daxx interactions. Future studies regarding putative covalent modification of Daxx by sentrin with Ubc9 and the mechanism of nuclear localization of Daxx would help to understand the regulation by sentrin upon Daxx.

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