Communication

Preferential Modification of Nuclear Proteins by a Novel Ubiquitin-like Molecule*

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Sentrin is a novel ubiquitin-like protein that protects cells against both anti-Fas and tumor necrosis factor-induced cell death. Antiserum recognizing the N terminus of sentrin revealed the presence of a 18-kDa sentrin monomer, a 90-kDa band (p90), and multiple high molecular mass bands. Because sentrin possesses the conserved Gly-Gly residues near the C terminus, it is likely that these additional bands represent conjugation of sentrin to other proteins in a manner that is similar to the ubiquitination pathway. Transient expression of hemagglutinin epitope-tagged sentrin mutants in COS cells demonstrated that the sentrin C terminus is cleaved, which allows it to be conjugated to other proteins via the conserved C-terminal Gly residue. Immunocytochemical staining and cell fractionation analysis demonstrated that sentrin monomer is localized predominantly to the cytosol. However, p90 and the majority of sentrinized proteins appeared to be localized to the nucleus. When the conserved Gly-Gly residues of sentrin were changed to Gly-Ala, only sentrin monomer and p90 but not the high molecular mass bands were observed. Thus, p90 generation appears to be required for the formation of high molecular mass bands in the nucleus. Taken together, sentrinization represents a novel pathway for nuclear protein modification, which is distinct from ubiquitination.

Sentrin was originally isolated in a yeast two hybrid screen using the death domain of Fas as bait (1). It also interacts with tumor necrosis factor (TNF) receptor 1 death domain but not with the death domain of FADD/MORT1 or CD40. When over-expressed in mammalian cells, sentrin protects cells against both anti-Fas and TNF-induced cell death. The mechanism of action of sentrin has not been clearly elucidated. Sentrin could block cell death signaling by blocking the assembly of the death-inducing signal complex. Alternatively, due to its homology to ubiquitin (18% identical and 48% similar), sentrin could exert its anti-death effect through modification of other proteins in a process similar to ubiquitination.

Protein modification by ubiquitin is critical for targeting proteins to be degraded by proteasomes (2–4). Conjugation of ubiquitin to other proteins requires initial activation of the conserved C-terminal Gly residue catalyzed by a specific ubiquitin-activating enzyme, E1. An intermediate, ubiquitin adenylation, is formed by displacement of PPI from ATP. Ubiquitin adenyylate is then transferred to a thiol site in E1 with release of AMP. Next, ubiquitin is transferred to a family of ubiquitin carrier proteins, E2, through transacylation. Finally, ubiquitin is transferred from E2 to its target protein through an isopeptide linkage with the ε-amino group of the Lys residue of the target protein. The transfer of ubiquitin from E2 to the target protein may require the participation of a ligase, E3. The internal Lys of ubiquitin, in particular Lys48, can also be modified by another ubiquitin to form mult ubiquitin chains that may be crucial for proteosome recognition (5). In recent years, ubiquitination has been shown to play a critical role in antigen processing, in the regulation of cell cycle, in receptor endocytosis, and in signal transduction (6–8).

Ubiquitin is not the only molecular tag for protein modification. Another ubiquitin-like protein, UCRP, has been shown to be conjugated to a large number of intracellular proteins (9). UCRP contains two ubiquitin domains and is inducible by type I interferons. There is evidence for a distinct pathway of UCRP conjugation that is parallel to ubiquitination (10). In this communication, we show that sentrin is another mammalian ubiquitin-like protein that can be conjugated to other proteins in a process analogous to ubiquitination. We show that the C terminus of sentrin is efficiently processed, which allows for subsequent protein conjugation. Furthermore, limited numbers of nuclear proteins are modified by sentrin, which is clearly distinct from ubiquitination. Remarkably, the presence of a sentrin-modified p90 appears to be a prerequisite for sentrin modification of nuclear proteins to occur.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Raji, Jurkat, HL60, SW837, and SK-N-SH were purchased from American Type Culture Collection (Rockville, MD). BJAB and COS-7 cells were generous gifts from Drs. Fred Wang and Dr. Steve Goldberg of Harvard Medical School. Cells were seeded in RPMI 1640 medium or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies—12CA5 (Boehringer Mannheim, Indianapolis, IN) and 16B12 (BAbCo, Richmond, CA) are mouse monoclonal antibodies (mAbs) to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA). The rabbit polyclonal anti-sentrin antiserum was generated by immunization with a peptide corresponding to amino acids 1–21 at the N terminus of sentrin. The antiserum was incubated overnight with beads coated with MBP or MBP-sentrin (1). The preabsorbed supernatant was used for Western blotting as described below.

Western Blotting—3 μl of total cell lysate (equivalent to 1 × 10^6 cells) was loaded on each lane of 10% or 12% polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane. Immobilon P (Millipore, Bedford, MA). Western blotting was performed using ECL detection system (Amersham Corp.) protocol. Horseradish peroxidase-conjugated antibodies against mouse IgG or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were added at a 1:5000 dilution in Odyssey blocking buffer. Nonspecific binding was blocked using Odyssey blocking solution. After washing in Odyssey washing solution, horseradish peroxidase-conjugated secondary antibodies were added. After further washing, the signal was visualized using a Odyssey infrared imaging system.

The abbreviations used are: TNF, tumor necrosis factor; HA, hemagglutinin epitope; MBP, maltose binding protein; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; mAb, monoclonal antibody; HA-Ubiquitin, HA-tagged ubiquitin; HA-Sentrin, HA-tagged sentrin.

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Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies.

**Plasmid Construction and Transfection**—To express HA-tagged proteins in COS-M6 cells, two vectors for N-terminal tagging (pDNA3/HA-N) and C-terminal tagging (pDNA3/HA-C) were constructed. HA adapter duplexes were inserted into pCDNA3 (Invitrogen, San Diego, CA) for vector construction. cDNAs of sentrin mutants were generated by polymerase chain reaction using appropriate primers followed by ligation with the vector, pDNA3/HA-N or pDNA3/HA-C. The insert sequences were confirmed by direct DNA sequencing. COS-M6 cells were transfected with LipofectAMINE (Life Technologies, Inc.) using the manufacturer’s recommendation. Transfected cells were harvested for Western blotting or immunostaining 16 h after transfection.

**Immunostaining**—Immunocytochemical staining was performed by the avidin-biotin-horseradish peroxidase complex (ABC-horseradish peroxidase) method using the VECTASTAIN ABC kit system (Vector, Burlingame, CA) as described previously (11). Transfected COS-M6 cells grown on a coverslip were fixed in 3% paraformaldehyde solution for 20 min and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. After washing, fixed cells were incubated with anti-HA antibody (16B12), followed by the incubation with biotinylated antimouse IgG and with ABC reagent (avidin-biotin-horseradish peroxidase complex). The final enzymatic disclosing procedure was performed as reported previously (11).

**Subcellular Fractionation**—Transfected COS-M6 cells were subfractionated as follows. To prepare S100 and P100, 3 × 10^7 cells were washed with phosphate-buffered saline, resuspended in 2 ml of hypotonic lysis buffer (5 mM Tris-HCl [pH 7.4], 2.5 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1.5 μg/ml pepstatin), and incubated on ice for 15 min to swell the cells. Cell suspension was homogenized by using a Dounce homogenizer followed by microscopical evaluation. The homogenate was centrifuged at 1,000 × g for 3 min to remove nucleus and undisrupted cells. Supernatant was centrifuged at 100,000 × g for 1 h. The pellet was solubilized with 200 μl of 2% SDS treating solution and used as the P100 fraction. The supernatant was concentrated with Centricon-10 (Amicon, Beverly, MA) up to 100 μl of the volume, mixed with 100 μl of 4% SDS treating solution, and used as S100 fraction. For the preparation of nuclear fraction, 3 × 10^7 cells were washed with phosphate-buffered saline, resuspended in 2 ml of hypotonic lysis buffer, and incubated on ice for 15 min followed by the Dounce homogenization. The homogenate was overlaid on 5 ml of lysis buffer containing 0.5 mM sucrose and centrifuged at 3,000 × g for 10 min. The pellet was solubilized with 200 μl of 2% SDS treating solution and used as nuclear fraction.

**RESULTS**

**Detection of Sentrin Monomer, p90, and High Molecular Mass Bands by Anti-Immunostaining Specific for the N Terminus of Sentrin**—Sentrin is a novel protein that contains an ubiquitin domain (residues 22–97) (1). In the ubiquitin domain, sentrin is 18% identical and 48% similar to ubiquitin. In contrast to ubiquitin, sentrin contains extra 21 amino acids at the N terminus and 4 more amino acids at the C terminus (Fig. 1). To study the expression of sentrin in cells, polyclonal antiserum against the N-terminal 21 amino acids was generated and put to use in a Western blot analysis. The antiserum was preabsorbed with either MBP or MBP-sentrin to demonstrate specificity of the immunoreactivity to sentrin. As shown in Fig. 2, an 18-kDa band specific for sentrin was observed in SK-N-SH, a neuroblastoma cell line. This 18-kDa bands most likely represents the sentrin monomer. However, the 18-kDa band could not be clearly detected in other cell lines. This could be due to rapid turnover of the sentrin monomer or rapid conjugation of sentrin to other proteins (see below). In addition to the 18-kDa band, a prominent 90-kDa band (p90) and a series of high molecular mass bands were observed in all cell lines. This was not unexpected because sentrin possessed the invariant Gly-Gly residues near the C terminus that would allow it to be conjugated to other proteins in a process analogous to ubiquitination (3, 4).

**Detection of Sentrin Monomer, p90, and High Molecular Mass Bands in a COS Cell Expression System**—To study the biochemistry of sentrin modification in more detail, a COS cell expression system using HA-tagged sentrin mutants was utilized. Briefly, HA-tagged sentrin mutant was transfected into COS cells by liposome-mediated transfection, and total cell lysates were prepared 16 h after transfection for Western blot detection using either anti-HA antibody or anti-sentrin antiserum. HA-tagged ubiquitin (HA-Ubiquitin) was used as a control. As shown in Fig. 3A, Western blot analysis of lysate prepared from COS cells expressing HA-Ubiquitin revealed a ladder of ubiquitin monomer, multimers, and ubiquitin-conjugated proteins. This pattern of ubiquitination has been seen previously in the yeast and in mammalian cells (3, 4). In contrast, HA-Sentrin expressing cell lysate revealed only the 18-kDa sentrin monomer, p90, and higher molecular mass sentrin conjugates. Thus, the COS cell transfection system yields results similar to that detected by polyclonal antisemur shown in Fig. 2. In addition, the COS cell transfection system allowed clear detection of the unconjugated monomer.

**The Gly-Gly Residues at the C Terminus of Sentrin Are Essential for Conjugating Sentrin to Other Proteins**—The C terminus of sentrin has four amino acids (His-Ser-Thr-Val) that follows the invariant Gly-Gly residues (1). It has been shown that activation of the Gly residue is critical for transfer of ubiquitin to the ubiquitin conjugating enzymes and eventually to proteins (3, 4). In order for sentrin to serve in a conjugation pathway analogous to that of ubiquitin, the C-terminal four amino acids have to be removed. To address the question of C-terminal processing, a sentrin construct with the HA-tag attached to the C terminus was made. When Sen-GGHSTV-HA was transfected into COS cells, sentrin monomer could not be detected with anti-HA mAb but still could be detected by anti-sentrin antisemur, suggesting that C-terminal HA-tag had been cleaved (Fig. 3B, lane 4). When the C-terminal four amino acids were removed (HA-Sen-GG), the expression pattern (monomer, p90, and high molecular mass bands) was similar to that of HA-Sen-GGHSTV transfactant (Fig. 3B, lane 5). Removal of the invariant Gly residue (HA-Sen-G) completely abolished the expression of p90 and the high molecular mass bands (data not shown). Thus, the presence of the C-terminal...
Gly^97 is essential for conjugation of sentrin to other proteins.

p90 Is a Critical Intermediate in the Formation of High Molecular Mass Bands—In the yeast, mutation of the C-terminal Gly-Gly of ubiquitin to Gly-Ala has been shown to impair hydrolase activity and cause irreversible conjugation of ubiquitin to other proteins (12). When a similar mutation was made in sentrin (HA-Sen-GAHSTV), only the monomer and p90 were observed (Fig. 3B, lane 7). The expression of high molecular mass bands had significantly decreased. Similar results were obtained from a second construct, HA-Sen-GA (Fig. 3B, lane 6). However, in the HA-Sen-GA lysate, a ~100-kDa band (p100) was also observed. These results suggest that Gly-Gly to Gly-Ala mutation in the sentrin molecule has a profound effect on either the processing or conjugation of sentrin to other proteins. In fact, only the sentrin monomer and p90 were consistently observed. These observations suggest that p90 is a key intermediate in the conjugation of sentrin to other high molecular mass proteins (see discussion).

Subcellular Localization of Sentrin Monomer, p90, and High Molecular Mass Sentrinized Proteins—The subcellular localization of sentrin and sentrinized proteins was determined next. COS cells were transfected with either HA-Ubiquitin or HA-Sentrin cDNA-containing plasmids as described previously, fixed, permeabilized, and stained with anti-HA mAb. As shown in Fig. 4A, HA-Ubiquitin could be detected both in the cytosol and the nucleus. In contrast, HA-Sentrin is mostly restricted to the nucleus. HA-Sentrin transfected COS cells were then fractionated into cytosolic (S100) and nuclear fractions and immunoblotted with anti-HA mAb. As shown in Fig. 4B, the high molecular mass bands were highly enriched in the nuclear fraction. p90 was mostly associated with the nuclear fraction, and the sentrin monomers were seen mostly in the cytosol.

**DISCUSSION**

In this communication, we show that sentrin can be conjugated to other proteins in a manner similar to the process of ubiquitination. Moreover, only a limited number of cellular proteins (p90 and the high molecular mass bands) are modified by sentrin. Remarkably, these sentrinized proteins appear to localize predominately to the nucleus.

Using antiserum specific for the N terminus of sentrin, we have shown that sentrin monomer is expressed at low levels in SK-N-SH cells but is not detectable in other cell types (Fig. 2). To study the processing of sentrin monomer, a COS cell expression system was utilized. Plasmids containing HA-tagged sentrin cDNA inserts were transfected into COS cells, and the tagged proteins were detected by Western blot analysis. The HA-tag was placed either in the N or C terminus of wild type sentrin or mutant sentrin. As shown in Fig. 3B, the C terminus of sentrin is efficiently processed in the transfected cells. Moreover, our results clearly demonstrate the requirement of the C-terminal Gly^97 residue for the formation of p90 and high molecular mass bands. Taken together, the processing of the C terminus of sentrin is analogous to the processing of all natural ubiquitin fusion protein by C-terminal hydrolases (S).

The HA-Sen-GAHSTV mutant is informative because only the sentrin monomer and p90 were detected in the lysate of transfected cells (Fig. 3B). Similar results were also seen in the HA-Sen-GA mutant except that an additional band, p100, is observed. Thus, it appears that formation of high molecular mass sentrinized proteins requires the formation of p90. In other words, p90 may be a key intermediate in the formation of high molecular mass bands. However, the precise mechanism awaits a more detail examination of the enzymology of the
conjugation process for sentrin. While this manuscript was being prepared, Matunis et al. (13) and Mahajan et al. (14) reported that a novel ubiquitin-like protein (GMP1/SUMO-1) is covalently attached to Ran-GTP, a 70-kDa Ras-like GTPase required for the bidirectional transport of proteins and ribonucleoproteins across the nuclear pore complex. Remarkably, GMP1/SUMO-1 is identical to sentrin. We also have evidence for the presence of p70 (unmodified RanGAP1) and p90 (presumably sentrinized RanGAP1) in COS cells transiently transfected with an HA-tagged RanGAP1 plasmid.2 At present, we cannot be completely certain that the p90 detected in this communication is identical to sentrinized (or GMP1-modified) RanGAP1. However, the similarity is compelling enough to allow us to make this temporary assignment.

RanGAP1 is a homologue of the murine Fug1 (15) and Saccharomyces cerevisiae and Schizosaccharomyces pombe Rna1p (16, 17). Unmodified RanGAP1 is localized in the cytosol but excluded from the nucleus (18). Modification of RanGAP1 by GMP1 (sentrin) is essential for its translocation to the cytoplasmic fiber of the nuclear pore complex (13). Thus, sentrinization of RanGAP1 is a crucial step in nuclear translocation and should have important implications for nucleocytoplasmic transport (19). Absence of high molecular mass sentrinized proteins in the HA-Sen-GAHSTV mutant is entirely consistent with the hypothesis that p90 (sentrinized RanGAP1) plays a critical role in the delivery of sentrin to the nucleus for modification of nucleoproteins. A hydrolase activity associated with the nuclear pore complex that releases sentrin from sentrinized proteins, such as the Fas, TNF receptor 1, RAD51, RAD52, and PML, which could be modified by sentrin. It is also possible that RAD51, RAD52, and PML are not themselves sentrinized but rather bind to other sentrinized proteins through noncovalent interaction. It is of interest to note that a yeast homologue of sentrin, smt3, is capable of correcting a conditional lethal mit2 mutation, which has increased mitotic chromosome instability (22, 23). This is consistent with the finding that sentrinized RanGAP1 interacts with the mitotic spindle apparatus during mitosis (13). These observations further underlie the importance of sentrin modification.

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