Post-translational modification marked by the covalent attachment of the ubiquitin-like protein SUMO-1/SMT3C has been implicated in a wide variety of cellular processes. Recently, two cDNAs encoding proteins related to SUMO-1 have been identified in human and mouse. The functions and regulation of these proteins, known as SUMO-2/SMT3A and SUMO-3/SMT3B, remain largely uncharacterized. We describe herein quantitative and qualitative distinctions between SUMO-1 and SUMO-2/3 in vertebrate cells. Much of this was accomplished through the application of an antibody that recognizes SUMO-2 and -3, but not SUMO-1. This antibody detected multiple SUMO-2/3-modified proteins and revealed that, together, SUMO-2 and -3 constitute a greater percentage of total cellular protein modification than does SUMO-1. Intriguingly, we found that there was a large pool of free, non-conjugated SUMO-2/3 and that the conjugation of SUMO-2/3 to high molecular mass proteins was induced when the cells were subjected to protein-damaging stimuli such as acute temperature fluctuation. In addition, we demonstrated that SUMO-2/3 conjugated poorly, if at all, to a major SUMO-1 substrate, the Ran GTPase-activating protein RanGAP1. Together, these results support the concept of important distinctions between the SUMO-2/3 and SUMO-1 conjugation pathways and suggest a role for SUMO-2/3 in the cellular responses to environmental stress.

SUMO-1 is a highly conserved, small ubiquitin-related modifier that has been shown to be covalently conjugated to a variety of cellular proteins (1–4). Like ubiquitin, SUMO-1 is believed to form an isopeptide bond between the carboxyl terminus of SUMO-1 and a lysine side chain(s) of the target protein (2, 3, 5). The conjugation of SUMO-1 to cellular proteins has been implicated in multiple vital cellular processes, including nuclear transport, cell cycle control, oncogenesis, inflammation, and the response to virus infection (6–8). It has been proposed that SUMO-1 conjugation may function antagonistically to ubiquitin conjugation (9) and/or that SUMO-1 conjugation may regulate the target protein’s interaction with other cellular components (5, 10, 11).

Among known SUMO-1 substrates in vertebrate species, the Ran GTPase-activating protein RanGAP1 is the most abundant and best characterized. It is a highly conserved protein that enhances GTP hydrolysis on Ran, a Ras-related small nuclear GTP-binding protein required for nucleocytoplasmic trafficking (12, 13). It has been demonstrated both in vitro and in vivo that a single lysine residue at position 526 in the C terminus of mouse RanGAP1 is modified by SUMO-1 (5, 10). A large fraction of SUMO-modified RanGAP1 appears to be tightly associated with the nuclear envelope via interaction with RanBP2/Nup358, a component of cytoplasmic filaments in the nuclear pore complex (NPC) (1) (1, 4, 14, 15). Unmodified RanGAP1 is present in the cytoplasm, suggesting that the conjugation of a ubiquitin-like moiety at the C-terminal domain may target RanGAP1 to the NPC (5, 10).

Two cDNAs coding for proteins related to SUMO-1 have been isolated from human and mouse cDNA libraries and are referred to as SUMO-2/SMT3A and SUMO-3/SMT3B (6, 16, 17). Both transcripts are expressed in a wide range of tissues and cell types, suggesting that their gene products play a role in some fundamental cellular processes. A comparison of the amino acid sequences of SUMO-1, -2, and -3 has revealed that SUMO-1 shares 48% identity with SUMO-2 and 46% identity with SUMO-3. Since SUMO-2 and -3 share 95% identity, it is reasonable to group SUMO-2 and -3 into a subfamily distinct from SUMO-1. It has been previously reported that both SUMO-2 and -3 could be transferred to other proteins in a pattern similar to that of SUMO-1 when SUMO-2/3 was transiently overexpressed in mammalian culture cells (18, 19). This result suggested that SUMO-2/3 might function in a capacity similar to that of SUMO-1. Very little is known, however, about the specific function of SUMO-2/3 and mechanistic differences that could support cellular distinctions between two SUMO subfamilies.

To gain a better understanding of the role of SUMO-2/3 and the differential regulation of SUMO-2/3 versus SUMO-1 conjugation in vertebrate cells, we have begun to characterize SUMO-2/3 at the protein level in vivo. We describe herein the development of an antibody specific to SUMO-2 and -3. Using this antibody and a complementary SUMO-1-specific antibody, we found that RanGAP1 was preferentially modified by SUMO-1, but very poorly by SUMO-2/3, despite a higher concentration of SUMO-2/3 than SUMO-1 in intracellular pools. We also demonstrated that the SUMO-2/3 conjugation pathway could be up-regulated by several protein-damaging stimuli. This is the first evidence for the involvement of a ubiquitin-like protein modification in cellular stress responses. Based on these results, we propose that there is distinct regulation of SUMO-2/3 modification as compared with SUMO-1 modification and that the SUMO-2/3 pathway may constitute an element of the cellular response to environmental stress.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Antibodies**—Human SUMO-1 cDNA and GST-SUMO-1 constructs have been described previously (20). SUMO-2 and SUMO-3 cDNA constructs have been described previously (21, 22). The abbreviations used are: NPC, nuclear pore complex; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ND10, nuclear domain 10.
-3 C-terminal deletion mutants, SUMO-2-(1–92) and SUMO-3-(1–93) were generated by polymerase chain reaction from human cDNA libraries and cloned into the pGEX vector (Amersham Pharmacia Biotech) for expression of GST fusion protein in a bacterial system or into the pEGFP-C1 vector (CLONTECH, Palo Alto, CA) for transient expression of green fluorescent protein (GFP) fusion product in a mammalian cell culture system. The GST-ubiquitin construct was described previously (20). Purified rabbit ubiquitin was purchased from Sigma. Anti-SUMO-1 and anti-RanGAP1 mouse monoclonal antibodies (21C7 and 19C7, respectively) were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Anti-RanBP2 polyclonal antibody raised in guinea pig was described previously (14). Horse serum peroxidase-conjugated secondary antibodies for immunoblot analysis were from Amersham Pharmacia Biotech. Rhodamine- and fluorescein-conjugated secondary antibodies for indirect immunofluorescence were obtained from Rockland Inc. (Gilbertsville, PA).

Antibody Preparation—The expression and purification of GST-SUMO-2 fusion protein were carried out as described previously (21). The purified protein was incubated with thrombin to cleave SUMO-2 from the GST moiety, followed by 4–20% gradient polyacrylamide gel electrophoresis (Novex, San Diego, CA) to separate the SUMO-2 and GST polypeptides. The 18-kDa band containing SUMO-2 was visualized by Coomassie Brilliant Blue, excised, and used to elicit antibodies in two rabbits (Research Genetics, Huntsville, AL). The antiserum from one of these rabbits was used for the studies described in this work.

Cell Extracts—COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics at 37 °C in a 5% CO2 incubator. Cells were harvested by gentle trypsinization and centrifugation. After washing the cells and counting in phosphate-buffered saline (PBS), the cells were mixed in SDS sample buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol. Fractionated interphase extracts were prepared as described previously (21, 22). The high speed supernatant that contained membrane-free soluble proteins is referred to throughout as the Xenopus egg extract.

Stress and Drug Treatments—Cells were harvested by gentle trypsinization and centrifugation. After counting, the cells (~10^7) were mixed in 100 μl of culture medium and subjected to one of the following stimuli: heat shock (10 min at 43 °C, unless stated otherwise), osmotic stress (3% sodium phosphate buffer, pH 7.2, containing 0.7 M NaCl for 15 min at 37 °C), oxidative stress (100 mM H2O2 for 20 min at 37 °C), or exposure to 7% ethanol (20 min at 37 °C).

Immunoblot and Immunoprecipitation Analyses—For immunoblot analysis, proteins were transferred to nitrocellulose membranes either in NuPAGE transfer buffer (Novex) at 25 V for 60 min for high molecular mass proteins (<30 kDa) or in 20 mM phosphate buffer at 50 V for 50 min for low molecular mass proteins (<30 kDa). Two different conditions were used because of the difficulty in transferring high and low molecular mass proteins with equal efficiency; when high molecular mass proteins were transferred well, the low molecular mass proteins were maintained in the membrane with less efficiency and vice versa. This unequal transfer efficiency can give a false impression of the relative intensity of immunoreactive bands. For example, free SUMO-1 and -2 are at ~18 kDa were not detectable upon blotting with NuPAGE buffer (data not shown), suggesting that these 18-kDa proteins are poorly immobilized on the membrane under NuPAGE transfer conditions. On the other hand, the signals, from high molecular mass SUMO-conjugated proteins in the phosphate buffer system were not as strong as the signals in the NuPAGE system (data not shown). We tested several other transfer conditions and found that phosphate buffer is reasonably efficient in transferring a wide range of proteins. The immunoblot data described in this study were obtained using phosphate buffer transfer conditions unless otherwise stated. Anti-SUMO-1 and anti-SUMO-2/3 antibodies were diluted 1:500 and 1:3000, respectively. Other antibodies were diluted 1:1000 unless otherwise stated. Detection of the signals in the immunoblot was carried out using the SuperSignal chemiluminescent substrate system (Pierce). To enrich for the SUMO-1-conjugated form of RanGAP1 from Xenopus egg extracts, immunoprecipitation was performed using anti-RanBP2 antibody following the detailed protocol described previously (14, 15).

| Presence of the SUMO-2 homolog in various vertebrate and insect species |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Human           | Mouse       | Zebrafish   | D. melanogaster | B. mori | S. cerevisiae |
| Human cDNA clonesa | X99584 | AF63847 | AI031364 | AI477817 | AF0553083 | AI004547 | U33057 |
| Identity to human SUMO-2 (%) | 48 | 49 | 48 | 47 | 52 | 54 | 48 |

a GenBank™ accession numbers of the representative cDNA clones are indicated.

Functional Heterogeneity of SUMO-1 versus SUMO-2/3

**RESULTS**

Presence of SUMO-2/3 Subfamily Members in Various Vertebrate and Insect Species—It has been reported that there are two human cDNAs encoding for proteins that have ~50% identity to human SUMO-1 (16). Because these predicted proteins, designated SUMO-2/SMT3A and SUMO-3/SMT3B, are ~50% identical to each other, we consider that they belong to the same subfamily and designate this subfamily as SUMO-2/3. The cDNAs encoding proteins homologous to human SUMO-2/3 were also isolated from mouse cDNA libraries (17), indicating the presence of two distinct SUMO subfamilies, SUMO-1 and -2/3, in both human and mouse. Thus, we first asked whether the two SUMO subfamilies are present in other vertebrates and in invertebrate species. Using the basic local alignment search tool algorithm BLAST, we found cDNAs encoding proteins with high homology to human SUMO-1 in the South African clawed toad (Xenopus laevis) (20) and the zebrafish (Danio reio; GenBank™ accession number AI545352). We also detected cDNAs encoding proteins with high homology to human SUMO-2 in these species (Table 1). Our data base search emphasized that the two SUMO subfamilies are widely distrib-
Functional Heterogeneity of SUMO-1 versus SUMO-2/3

Fig. 1. SUMO-2/3-specific rabbit polyclonal antibody. A–D, specificity against SUMO-2 and -3. Purified recombinant SUMO-2 was used to elicit rabbit anti-SUMO-2 antisera. 0.1 μg of purified GST-SUMO-1 (lane 1), GST-SUMO-2 (lane 2), and GST-SUMO-3 (lane 3) was subjected to 4–20% SDS-PAGE followed by immunoblotting. Lanes 1–3 show the transfer of proteins. Other blots were probed with anti-SUMO-1 monoclonal antibody (mAb) 21C7 (B), preimmune serum (C), or serum from a rabbit immunized against SUMO-2 (D). 10-kDa ladder protein markers are indicated on the left (lane M). E–G, absorption of the SUMO-2/3-specific antibody. The immune serum was preincubated with GST-SUMO-1 (E), GST-SUMO-2 (F), or GST-SUMO-3 (G), followed by immunoblot analysis using a membrane identical to that used in A–D. H and I, anti-SUMO-2/3 antibody does not cross-react with ubiquitin. 2 μg of purified ubiquitin (lane 1), 0.5 μg of purified GST-ubiquitin (lane 2), and 0.5 μg of GST-SUMO-3 (lane 3) were subjected to 4–20% SDS-PAGE followed by immunoblotting. A parallel blot was stained with Ponceau S (H) to show the transfer of proteins. Another blot was probed with anti-SUMO-2/3 antibody (I). The positions of ubiquitin, GST-ubiquitin, and GST-SUMO-3 are indicated on the left.

Development of Antibody to SUMO-2/3—To aid in our study of the function and regulation of SUMO-2/3 at the protein level, we raised rabbit polyclonal antibodies to SUMO-2. Antibody was raised against bacterially produced recombinant polypeptide corresponding to amino acids 1–92 of human SUMO-2. To establish the specificity of this antibody, we probed immunoblots of GST-SUMO-1, GST-SUMO-2, and GST-SUMO-3, as shown in Fig. 1D, the immune serum from one of the immunized rabbits recognized GST-SUMO-2 and GST-SUMO-3, but not GST-SUMO-1. No significant signal could be detected when the identical blot was probed with the preimmune serum from the same rabbit (Fig. 1C). When the immune serum was preincubated with Sepharose beads bearing either GST-SUMO-2 or GST-SUMO-3, the depleted serum did not recognize SUMO-2/3 in the immunoblot, whereas preincubation of the serum with GST-SUMO-1 did not alter the pattern of the signals (Fig. 1, E–G). These results indicate that this particular immune serum contains antibodies that specifically recognize epitopes in SUMO-2 and -3 that are not present in SUMO-1; therefore, we refer to this immune serum as anti-SUMO-2/3 antibody.

Given that the amino acid sequences of SUMO-2 and -3 are almost identical (95% identity), the fact that this serum did not distinguish between the SUMO-2/3 family members under the conditions we employed throughout this investigation was expected. We also probed the immunoblot containing ubiquitin and GST-ubiquitin to exclude the possibility that anti-SUMO-2/3 antibody cross-reacts with ubiquitin. As shown in Fig. 1 (H and I), this antibody did not recognize either ubiquitin or GST-ubiquitin under our experimental conditions.

For the detection of SUMO-1, we used monoclonal antibody 21C7, which was generated from BALB/c mice immunized against recombinant human SUMO-1 (1). Monoclonal antibody 21C7 detected only GST-SUMO-1 in immunoblots containing all three GST-SUMO proteins (Fig. 1E), indicating that the epitope recognized by the monoclonal antibody is absent in SUMO-2/3. Therefore, this monoclonal antibody is referred to as anti-SUMO-1 antibody in this study.

Abundance of SUMO-2/3 Versus SUMO-1—Using the SUMO-2/3-specific and SUMO-1-specific antibodies, we first compared the relative amount of SUMO-2/3 versus SUMO-1 in COS-7 cells by semiquantitative immunoblot assay using GST-SUMO-1 and GST-SUMO-3 as standards. As shown in Fig. 2, we determined conditions under which the assay exhibited fair linearity (see “Experimental Procedures” in detail). Under these conditions, we compared the signal intensity of cell lysates in SUMO-1 versus SUMO-2/3 blots.

Judging by apparent molecular mass, an 18-kDa band appeared to represent a non-conjugated pool of either SUMO-1 or SUMO-2/3, whereas bands migrating higher than 18 kDa most likely contain proteins conjugated with either SUMO-1 or SUMO-2/3. The SUMO-2/3 blot displayed a strong 18-kDa band, indicating that a large fraction of SUMO-2/3 is present as a “free” or non-conjugated form in vivo. On the other hand, a 90-kDa protein was detected as the dominant signal in the SUMO-1 blot, whereas the 18-kDa band was barely detectable. This observation suggests that most SUMO-1 exists as protein conjugate rather than in a free form in vivo and that a major target of SUMO-1 conjugation is a 90-kDa protein. Interestingly, the amount of non-conjugated SUMO-2/3 was ~50 times greater than that of non-conjugated SUMO-1 (Fig. 2C), indicating that the SUMO-2/3 modification system has a potentially larger capacity to modify cellular proteins than the SUMO-1 system. When the signal spanning from 18 kDa to the top of either lane 5 or 11 in A was measured and integrated, respectively, the total signal intensity of lane 11 in the SUMO-2/3 blot was ~4 times greater than that of lane 5 in the SUMO-1 blot (Fig. 2C). These results suggest that the total cellular pool of SUMO-2/3 is greater than that of SUMO-1.

Activation of SUMO-2/3 Conjugation by Acute Heat Fluctuation—To determine whether the large pool of free SUMO-2/3 is functional in terms of protein conjugation, we tested multiple stimuli, including anticancer drugs, enzyme inhibitors, and environmental stresses such as temperature fluctuation, for associated changes in SUMO-2/3 conjugation. As shown in Fig. 3, we found that acute temperature elevation was associated with a diminished pool of free SUMO-2/3 and an accumulation of high molecular mass SUMO-2/3 signals. Strikingly, the response to acute heat elevation was detected in as little as 5 min, suggesting the existence of a rapidly signaling cellular pathway that activates SUMO-2/3 conjugation in response to acute heat fluctuations.
Functional Heterogeneity of SUMO-1 versus SUMO-2/3

FIG. 2. SUMO-2/3 is more abundant than SUMO-1. A, semiquantitative immunoblot analysis of SUMO-2/3 versus SUMO-1. Exponentially growing COS-7 cell cultures were lysed directly in SDS sample buffer. Proteins from 1 × 10⁵ cells (lanes 5, 6, 11, and 12), purified GST-SUMO-1 (lanes 1–4), and GST-SUMO-3 (lanes 7–10) were separated by 4–20% SDS-PAGE and transferred to nitrocellulose membranes in phosphate buffer. Lanes 1 and 7 contain 0.44 ng, lanes 2 and 8 contain 1.33 ng, lanes 3 and 9 contain 4.0 ng, and lanes 4 and 10 contain 12 ng of either purified GST-SUMO-1 or GST-SUMO-3, respectively. Immunoblot analysis was performed using anti-SUMO-1 antibody (lanes 1–5) or anti-SUMO-2/3 antibody (lanes 7–11) as the primary antibody. The antibodies were detected by 125I-labeled protein G as described under “Experimental Procedures.” The arrowhead indicates bands at ~18 kDa that may represent the free, non-conjugated form of SUMO-2/3. The asterisk indicates a 50-kDa band nonspecifically detected in all lanes. 10-kDa protein size markers are shown on the left. B, the linearity of immunoblot assay. 125I-Labeled signals in lanes 1–4 for GST-SUMO-1 and lanes 7–10 for GST-SUMO-3 in A were quantified by InstantImager electronic autoradiography. The conditions we employed exhibited a fair linearity between the amount of GST proteins and the signal intensities. C, the relative amount of SUMO-2/3 versus SUMO-1. Signals in lanes 5 and 11 in A were measured and integrated, respectively. These values were considered to represent the total amount of SUMO-1 and -2/3, respectively, and were compared with each other. 18-kDa signals in lanes 5 and 11 were separated and also measured. These values were considered as amounts of free, non-conjugated forms of SUMO-1 and -2/3 for comparison with the total signal intensities. a.U., absorbance units.

fluctuation. In addition, we found that the ratio between free SUMO-2/3 and high molecular mass conjugates returned to its basal state when cell cultures were incubated at 37 °C for 60 min after acute heat stress (43 °C for 10 min) (Fig. 3, B and D). These results indicate that a substantial pool of free SUMO-2/3 is readily available for conjugation and imply that SUMO-2/3 is a reversible protein modifier in the context of heat shock. In contrast to SUMO-2/3, the pattern of SUMO-1 conjugation was not dramatically changed following heat shock, suggesting that activation of the SUMO-2/3 conjugation pathway is a more prominent feature of the cellular stress response than is that of the SUMO-1 pathway. In Fig. 3A, a 50-kDa band was observed in all lanes, and the intensity of the band appeared to be constant in the presence or absence of stimuli. We concluded that this 50-kDa band was nonspecific since the anti-SUMO-2/3 serum preincubated with either GST-SUMO-2 or GST-SUMO-3 also recognized the 50-kDa protein band (data not shown).

Given that heat stress induces denaturing of cellular proteins, we wondered whether other protein-damaging conditions would up-regulate protein modification by the SUMO-2/3 pathway. To begin investigating the response of the SUMO-2/3 pathway to various protein-damaging stresses, we monitored the activation of SUMO-2/3 conjugation during oxidative stress, ethanol addition, and osmotic stress (Fig. 4A). Immunoblot analysis of COS-7 cell extracts from cultures subjected to these stresses showed a marked accumulation of high molecular mass SUMO-2/3 conjugates and a diminished pool of free SUMO-2/3 (Fig. 4, A and B); osmotic stress induced less dramatic changes than the other stresses. We also found that the human leukemia cell line K562 responded to these stresses
Functional Heterogeneity of SUMO-1 versus SUMO-2/3

FIG. 4. Protein-damaging stimuli induce SUMO-2/3 conjugation. A, effect of different stresses on SUMO-2/3 conjugation. COS-7 cells were treated with no stress (Control), heat stress (43 °C for 10 min), oxidative stress (100 mM H2O2 for 20 min), ethanol (7% for 20 min), or osmotic stress (hypertonic stress, 0.7 M NaCl for 15 min). After treatments, proteins from 0.3 × 10⁶ cells were subjected to SDS-PAGE followed by immunoblot analysis using anti-SUMO-2/3 antibody. Heat shock, oxidative stress, and ethanol addition induced accumulation of high molecular mass SUMO-2/3 immunoreactive proteins. The arrowhead indicates the position of the non-conjugated form of SUMO-2/3 at 18 kDa. B, diminution of free SUMO-2/3 by protein-damaging stimuli. The 18-kDa bands in all lanes in A considered to represent the non-conjugated pool of SUMO-2/3 were measured, plotted, and compared with each other.

with a similar redistribution of SUMO-2/3 immunoreactivity (data not shown), suggesting that prompt conjugation of free SUMO-2/3 to target protein(s) may be a stress response common to many cell systems.

Preferential Modification of RanGAP1 by SUMO-1—As described above (Fig. 2), a distinct 90-kDa band was evident in immunoblot analysis of nonstimulated COS-7 cell cultures probed with anti-SUMO-1 antibody. Immunoreactivity at this apparent molecular mass was barely detectable in semiquantitative immunoblots probed with anti-SUMO-2/3 antibody. It has been well established that RanGAP1 (which migrates at 90 kDa in SDS-PAGE) is the most abundant SUMO-1 substrate (1, 4, 15), but we were unable to detect a parallel modification of 90-kDa RanGAP1 by SUMO-2/3 (Figs. 2 and 3). In overexposed immunoblots, a band reactive to anti-SUMO-2/3 antibody at a molecular mass approximately equivalent to that of SUMO-1-conjugated RanGAP1 (90 kDa) can be distinguished (Fig. 5A, arrowhead). We attempted to enrich for this 90-kDa SUMO-2/3 conjugate by immunoprecipitation using anti-RanGAP1 monoclonal antibody 19C7 (1), but the cross-reactive signal indicating a SUMO-2/3-modified 90-kDa form of RanGAP1 remained difficult to detect even in this enriched immunoprecipitate (data not shown). By comparing band intensities at 90 kDa in SUMO-1- versus SUMO-2/3-stained immunoblots (Fig. 5A), we estimated that SUMO-2/3-conjugated RanGAP1 constitutes not more than 5% of the amount of SUMO-1-modified RanGAP1. In addition to COS-7 extracts, we tested human HeLa, 293, SK-N-M, and NB4; mouse NIH-3T3 and liver; and Xenopus egg extracts to compare the relative amount of SUMO-2/3 versus SUMO-1-conjugated RanGAP1. None of these cell extracts contained a higher percentage of SUMO-2/3-conjugated RanGAP1 than did the COS-7 cells (data not shown), indicating that RanGAP1 is modified preferentially by SUMO-1, but very poorly, if at all, by SUMO-2/3 in many cell types.

Absence of SUMO-2/3 in the NPC/Nuclear Envelope—It has been proposed that SUMO-1 conjugation facilitates the localization of RanGAP1 to the NPC via interaction with RanBP2/Nup358, a component of cytoplasmic filaments in the NPC (5, 10). To test whether the limited cellular pool of SUMO-2/3-modified RanGAP1 is involved in the interaction between RanGAP1 and RanBP2, we performed three experiments. First, we
carried out indirect immunofluorescence of COS-7 cells permeabilized with digitonin prior to fixation and staining (1). Digitonin preferentially permeabilizes the plasma membranes of eukaryotic cells versus their nuclear envelopes. Thus, treatment of cells with digitonin before fixation removes large amounts of soluble proteins from the cells, allowing more sensitive detection of the NPC/nuclear envelope-associated proteins. When COS-7 cells that had been pretreated with digitonin were probed with anti-SUMO-2/3 antibody, there was no staining of the nuclear rim, whereas anti-RanGAP1 and anti-SUMO-1 antibodies clearly localized to the nuclear rim (Fig. 5B). Second, we expressed GFP-SUMO-1 or GFP-SUMO-2 fusion protein in COS-7 cells and observed a marked concentration of green fluorescence at the nuclear rim in GFP-SUMO-1-transfected cells, whereas there was no significant concentration of the signal at the nuclear rim in GFP-SUMO-2-transfected cells (data not shown). Third, using anti-RanBP2 antibodies, we co-immunoprecipitated RanGAP1 that associated with RanBP2 from Xenopus laevis egg extracts (14, 15) and analyzed the precipitated fraction by immunoblotting using anti-SUMO-1 or anti-SUMO-2/3 antibody. We were unable to detect a significant signal when this immunoblot was probed with anti-SUMO-2/3 antibody, despite the enrichment for RanBP2-associated RanGAP1 (data not shown). This result indicates that SUMO-1 is a dominant modifier of RanGAP1 and further implies that, if there is a minority complex containing SUMO-2/3-modified RanGAP1 and RanBP2, it must be unstable in Xenopus egg extracts. This result is not simply attributable to a lack of SUMO-2/3 in Xenopus egg extracts since we found a X. laevis cDNA coding for a SUMO-2 protein homolog (Table I). Also, we detected an 18-kDa protein that cross-reacted to anti-SUMO-2/3 antibody in Xenopus egg extracts (data not shown), suggesting the presence of a SUMO-2/3 homolog in Xenopus eggs. In sum, these results support the idea that RanGAP1 associated with the NPC/nuclear envelope is preferentially modified by SUMO-1 rather than by SUMO-2/3.

It should be noted that we observed an intense nuclear signal of SUMO-2/3 as well as some punctate concentrations in the nucleus (Fig. 5B); however, the number and size of these foci varied from cell to cell (data not shown). Intriguingly, some of the foci were stained by both anti-SUMO-1 and anti-SUMO-2/3 antibodies, indicating the colocalization of SUMO-1 and SUMO-2/3 in the digitonin-insoluble, nuclear punctate structures in interphase cells.

**DISCUSSION**

On the basis of sequence homology, the known small ubiquitin-related modifiers (SUMO) can be divided into two groups in vertebrates: one including human SUMO-1 and its interspecies homologs and the other comprising human SUMO-2, SUMO-3, and their counterparts in other species. Data base searches for proteins homologous to SUMO-2/3 revealed that SUMO-2/3 is highly conserved not only in vertebrate species, but also in insect species, suggesting a biologically significant role for the SUMO-2/3 conjugation pathway.

Using a newly developed anti-SUMO-2/3 antibody, we demonstrated that SUMO-2/3- and SUMO-2/3-conjugated proteins are present in vertebrate cells. One obvious difference between SUMO-1 and -2/3 was the presence of a large pool of free or non-conjugated SUMO-2/3 in COS-7 cells. This fraction of SUMO-2/3 appears to be readily available for conjugation reactions since its conjugation can be induced by cellular stresses such as acute heat elevation, oxidative stress, and ethanol addition, which are known to cause the accumulation of damaged proteins in the cells and to induce several stress-responsive kinase cascades (23, 24). Recently, we found that treatment by the peptide aldehyde protease inhibitor MG-132, which has been reported to induce protein damage (25), also effectively up-regulates the conjugation of SUMO-2/3 to a high molecular mass protein fraction (data not shown). We are in the process of investigating proteins specifically modified by SUMO-2/3 in a stress-responsive manner and the fate of such SUMO-2/3-modified proteins in stressed cells.

It has been reported that the ubiquitin pathway responds to a wide variety of protein-damaging and environmental stresses (26). Intriguingly, heat shock (27, 28) and oxidative (29) stresses have been known to induce the nearly quantitative conversion of free ubiquitin to the conjugated pool, reminiscent of the stress-induced SUMO-2/3 conjugation described in this study. Given that ubiquitin conjugation to damaged cellular proteins induces rapid degradation of the conjugates by 26 S proteasomes, the similar mode of stress response in the SUMO-2/3 pathway might represent a novel mechanism by which damaged proteins are identified and processed. For instance, SUMO-2/3 conjugation might stabilize the target protein by protecting against ubiquitin conjugation and the subsequent rapid proteasomal degradation. Recent observations suggest that IsBo, an inhibitor for the NF-κB transcription factor, can be conjugated with either ubiquitin or SUMO-1 on Lys21 (9). IsBo conjugated with SUMO-1 remains stable, but ubiquitin conjugates are subjected to rapid proteasomal degradation. Since modifications of IsBo with SUMO and ubiquitin are mutually exclusive, it is likely that these modifications functionally antagonize each other. Alternatively, SUMO-2/3 conjugation might enhance the denaturation process during stress and augment the ubiquitin-dependent degradation of such SUMO-2/3-conjugated, denatured/damaged protein by 26 S proteasomes. Conjugation by either SUMO-1 or SUMO-2/3, however, has not yet been reported to increase protein instability.

Whatever its role, our results suggest that the SUMO-2/3 conjugation pathway represents a new class of acute and reversible stress response. The rapid kinetics of SUMO-2/3 conjugation and deconjugation reveal highly dynamic features of the regulation of the SUMO-2/3 conjugation pathway in the context of environmental stress. A complete understanding of the SUMO-2/3 conjugation pathway in relation to environmental stress will require further information not only on the SUMO-2/3 conjugation substrate(s), but also on the regulation of the SUMO-2/3 conjugation/deconjugation enzymes during environmental stress.

Strikingly, the SUMO-1 and -2/3 modification pathways can be clearly distinguished by their differential modification of RanGAP1, which is a major SUMO-1 substrate, but is very poorly modified by SUMO-2/3. According to previously published reports, there is no indication of any ubiquitin-related polypeptide modification of RanGAP1 purified from mammalian cells and X. laevis egg extracts except SUMO-1 (1, 5, 14). These investigations of endogenous RanGAP1 support the hypothesis of selective modification of RanGAP1 by SUMO-1 in vivo. Others studies have shown, however, that RanGAP1 is efficiently modified by SUMO-2 and -3 as well as by SUMO-1 when cDNA carrying SUMO-2 or -3 is transiently overexpressed in mammalian cell culture (18, 19). Since we identified the preferential modification of RanGAP1 by SUMO-1 not only in COS-7 cells, but also in other cells such as human HeLa, 293, SK-N-M, and NB4 and mouse NIH-3T3 and liver, a more reasonable explanation might be that overproduction of either SUMO-2 or -3 by transient transfection perturbs the specificity of the normal modification system.

The mechanism that governs the specificity of modification between SUMO-1 and -2/3 is currently unexplained, although
three possibilities appear feasible. First, there may be two parallel enzymatic cascades present in cells that independently facilitate the modification of proteins with either the SUMO-1 or -2/3 family, respectively. For example, there is evidence for the existence of two slightly different isoforms of human Ubc9p (30) and three isoforms of mouse Ubc9p (31). Ubc9p is an enzyme whose activity has been demonstrated to be required for SUMO-1 conjugation to RanGAP1 (20, 32). It will be interesting to compare these isoforms in terms of their specificity for SUMO-1 or -2/3. Second, similar to the ubiquitin conjugation system, one might predict the existence of a ubiquitin-protein isopeptide ligase-like activity that docks Ubc9p with RanGAP1 and determines the specificity of SUMO-1 conjugation to RanGAP1. One likely candidate for this putative activity is RanBP2/Nup358, as it has been shown to interact with both Ubc9p and SUMO-1-modified RanGAP1 (14, 15). Third, SUMO-1 and -2/3 might both be conjugated to RanGAP1, followed by selective deconjugation of SUMO-2/3 from RanGAP1. This scenario would require SUMO-2/3-specific isopeptidase activity in the context of SUMO-1 modification of RanGAP1. The sulfhydryl proteases from yeast and bovine brain that exhibit SUMO-1-releasing activity from SUMO-1-conjugated RanGAP1 (31, 33) and the previously described deconjugation activity associated with the NPC fraction (1) represent likely candidates for the SUMO-2/3 isopeptidase, but preferential SUMO-2/3 deconjugation activity has not yet been identified.

Prolonged exposure of SUMO-2/3 immunoblots revealed multiple discrete minor bands in the range of 100 kDa (Fig. 5). Most of these immunoreactive proteins were absent in the immunoblot probed with anti-SUMO-1 antibody, suggesting that a number of cellular proteins may become selectively modified by SUMO-2/3 as opposed to SUMO-1. However, one might point out that some of the high molecular mass bands (>120 kDa) were similar in size to the high molecular mass bands that appeared in the immunoblot probed with anti-SUMO-1 antibody. Although the identities of these proteins remain to be established, some might represent common substrates for SUMO-1 and -2/3 (i.e. mixed conjugation products including both SUMO-1 and -2/3). This observation argues for the possibility of cross-talk or convergence of the SUMO-1 and -2/3 modification pathways, at least with regard to some common substrates.

Nuclear foci detected by anti-SUMO-1 antibody have been colocalized with the nuclear matrix-associated, large multiprotein complex known as nuclear domain 10 (ND10), nuclear bodies, or promyelocytic leukemia protein oncogenic domains (11, 34–36). The functions of ND10 are not well understood, but size, number, and composition appear to be regulated throughout the cell cycle and to respond to environmental stress, interferon, and infection of several DNA viruses. We found that the nuclear foci detected by anti-SUMO-2/3 antibody appeared to represent staining patterns exhibited in common with SUMO-1 (Fig. 5F). Currently, we observed that Asp203, which has been reported to affect the assembly/maintenance of ND10 (11), induced the accumulation of both SUMO-1 and -2/3 in the nuclear punctate structures (data not shown). These results suggest that SUMO-1 and -2/3 are components of ND10 and imply involvement of the SUMO-2/3 pathway in the assembly or function of ND10.

The data presented thus far indicate that although there is functional heterogeneity between the SUMO-1 and -2/3 subfamily members, it is currently unknown whether further functional heterogeneity might be present in the SUMO-2 versus SUMO-3 pathways. It will be important in the future to generate a monoclonal antibody that distinguishes between SUMO-2 and -3 subfamily members, but this is likely to be complicated by the high homology between SUMO-2 and -3.

In summary, we have generated specific antibodies to SUMO-2/3 and have found that a number of proteins exist in SUMO-2/3-conjugated forms. The combination of this antibody and anti-SUMO-1 antibody revealed several differences between the SUMO-1 and -2/3 modification pathways. First, SUMO-2/3 is present in greater abundance than SUMO-1, and there is a large pool of non-conjugated SUMO-2/3 in COS-7 cells. Remarkably, all pool of free SUMO-2/3 was promptly activated and incorporated into high molecular mass proteins by protein-damaging stresses such as heat shock. Second, RanGAP1 is preferentially modified by SUMO-1, but very poorly by SUMO-2/3 in vivo, and SUMO-1-modified RanGAP1 appears to localize more abundantly than the NPC/nuclear envelope than the little SUMO-2/3-modified RanGAP1 that may be present, if any. Collectively, these results imply that regulatory and functional heterogeneity between the SUMO-1 and -2/3 modification systems and suggest a role for SUMO-2/3 in the response to environmental stress.

Acknowledgments—We thank the Picower Institute for Medical Research and its members for supporting our research project. We thank Dr. Maria Ruggieri and Marc Symons for discussions. We are grateful to Drs. Richard Bucala and Kirk Manogue for helpful comments on the manuscript.

REFERENCES

1. Matunis, M. J., Coutavas, E., and Blobel, G. (1996) J. Cell Biol. 133, 1457–1470
2. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1997) EMBO J. 15, 5509–5519
3. Kamitani, T., Nguyen, H. P., and Yeh, E. T. (1997) J. Biol. Chem. 272, 14001–14004
4. Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) Cell 88, 97–107
5. Mahajan, R., Gerace, L., and Melchior, F. (1997) J. Cell Biol. 139, 259–270
6. Kamitani, T., Kito, K., and Yeh, E. T. (1999) J. Cell Biol. 147, 1161–1174
7. Kamitani, T., Kito, K., Nguyen, H. P., Fukuda-Kamitani, T., and Yeh, E. T. (1998) J. Biol. Chem. 273, 3117–3120
8. Kamitani, T., Kito, K., Nguyen, H. P., Fukuda-Kamitani, T., and Yeh, E. T. (1998) J. Biol. Chem. 273, 11349–11353
9. Saitoh, H., Sparrrow, D. B., Shiomi, T., Pu, R. T., Nishimoto, T., Mohun, T. J., and Dasso, M. (1998) Mol. Gen. Genet. 260, 499–509
10. Muller, S., Matunis, M. J., and Dejean, A. (1998) EMBO J. 17, 61–70
11. Biscoff, F. R., Krebber, H., Kemm, F. T., Hermes, I., and Pontoosting, H. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 1749–1753
12. Melchior, F., and Gerace, L. (1998) Trends Cell Biol. 8, 175–179
13. Saitoh, H., Cooke, C. A., Burgess, W. H., Kearnshaw, W. C., and Dasso, M. (1997) Mol. Cell Biol. 17, 1319–1334
14. Saitoh, H., Pu, R., Cavenagh, M., and Dasso, M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 3736–3741
15. Lapenta, V., Chiurazzi, P., van der Spek, P., Fizzi, A., Hanaoka, F., and Brahe, C. (1997) Genomics 40, 362–366
16. Chen, A., Mannen, H., and Li, S. S. (1998) Biochem. Mol. Biol. Int. 46, 1161–1174
17. Kamitani, T., Yeh, E. T. H., Strauss, J. F., and Maul, G. G. (1999) J. Biol. Chem. 274, 1621–1634

Access the most updated version of this article at http://www.jbc.org/content/275/9/6252

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 20 of which can be accessed free at http://www.jbc.org/content/275/9/6252.full.html#ref-list-1