The Small Ubiquitin-Like Modifier (SUMO) and SUMO-Conjugating System of *Chlamydomonas reinhardtii*

Ying Wang, Istvan Ladunga, Amy R. Miller, Kempton M. Horken, Thomas Plucinak, Donald P. Weeks and Cheryl P. Bailey

Department of Biochemistry and Center for Plant Science Innovation, University of Nebraska, Lincoln, Nebraska 68588-0664

Manuscript received March 10, 2008
Accepted for publication March 27, 2008

**ABSTRACT**

The availability of the complete DNA sequence of the *Chlamydomonas reinhardtii* genome and advanced computational biology tools has allowed elucidation and study of the small ubiquitin-like modifier (SUMO) system in this unicellular photosynthetic alga and model eukaryotic cell system. SUMO is a member of a ubiquitin-like protein superfamily that is covalently attached to target proteins as a post-translational modification to alter the localization, stability, and/or function of the target protein in response to changes in the cellular environment. Three SUMO homologs (*CrSUMO96, CrSUMO97*, and *CrSUMO148*) and three novel SUMO-related proteins (*CrSUMO-like89A, CrSUMO-like89B, and CrSUMO-like90*) were found by diverse gene predictions, hidden Markov models, and database search tools inferring from *Homo sapiens*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* SUMOs. Among them, *GtSUMO96*, which can be recognized by the *A. thaliana* anti-SUMO1 antibody, was studied in detail. Free *CrSUMO96* was purified by immunoprecipitation and identified by mass spectrometry analysis. A SUMO-conjugating enzyme (SCE) (**E2, Ubc9**) in *C. reinhardtii* was shown to be functional in an *Escherichia coli*-based *in vivo* chimeric SUMOylation system. Antibodies to *CrSUMO96* recognized free and conjugated forms of *CrSUMO96* in Western blot analysis of whole-cell extracts and nuclear localized SUMOylated proteins with *in situ* immunofluorescence. Western blot analysis showed a marked increase in SUMO conjugated proteins when the cells were subjected to environmental stresses, such as heat shock and osmotic stress. Related analyses revealed multiple potential ubiquitin genes along with two *Rub1* genes and one *Ufm1* gene in the *C. reinhardtii* genome.

**POST-TRANSLATIONAL** modification can regulate protein function and cellular processes in a rapid and reversible manner. In addition to protein modification by small molecules such as phosphate and carbohydrates, peptides and small proteins also serve as modifiers. The three most studied small polypeptides that covalently modify other cellular proteins are ubiquitin, small ubiquitin-like modifier (SUMO), and neural precursor cell-expressed developmentally down-regulated (Nedd)8 (Johnson 2004; Kerscher et al. 2006; Geiss-Friedlander and Melchior 2007; Palancake and Doyle 2008). Ubiquitin amino acid sequence is highly conserved and the conjugation of ubiquitin to target proteins usually, but not always, results in their degradation by the 26S proteasome (Pickart 2000, 2001, 2004). Nedd8 shares high similarity with ubiquitin (60% identity and 80% similarity), and the primary substrates for Nedd8 in yeast and mammalian cells are Cullin proteins that play an important role in ubiquitin-mediated proteolysis (Kamitani et al. 1997; Ye et al. 2000; Pan et al. 2004).

The three-dimensional (3-D) structure of human and yeast SUMO closely resembles that of ubiquitin (Melchior 2000; Hay 2001; Weissman 2001; Seeler and Dejean 2003; Johnson 2004). A prominent structural feature of SUMO is a long and highly flexible N terminus, which protrudes from the globular core of the protein. Despite the similarities in overall conformation, SUMO functions quite differently from ubiquitin. That is, SUMOylation often enables target proteins to participate in new and diverse cellular processes, including nuclear transport, transcriptional regulation, maintenance of genome integrity, and signal transduction (Seeler and Dejean 2003; Colby et al. 2006).

In yeast and invertebrates, a single SUMO gene has been identified and has been shown to be essential for viability in *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, while in *Schizosaccharomyces pombe*, mutants lacking the single SUMO gene remain viable, but suffer severe defects in genome maintenance (Tanaka et al. 1999; Li and Hochstrasser 2003; Broday et al. 2004). Organisms have different numbers of SUMO isoforms and...
some SUMO isoforms appear to fulfill specialized functions. In humans, four major SUMO family members have been described, namely SUMO-1 to -4 (Melchior 2000; Hay 2001; Guo et al. 2004). Human SUMO-2 and -3 share 95% identity and their conjugation is strongly induced in response to various stresses (Holmstrom et al. 2003). In Arabidopsis thaliana, eight genes encoding SUMOs have been described (Kurepa et al. 2003). Similarity analysis clustered these SUMO proteins into five subfamilies: SUMO1/2, SUMO3, SUMO5, SUMO4/6, and SUMO7/8. As A. thaliana SUMO1 amino acid sequence is equally related to human SUMO1-1, -2, and -3, it is difficult to group the A. thaliana SUMO proteins with animal and yeast homologs. As SUMOs from more plant and algal species are fully characterized, the relationship between SUMO sequence and function in plant biology likely will become clearer.

SUMOylation, the conjugation of SUMO peptide(s) to the target protein, results in an isopeptide bond between the C-terminal carboxyl group of a double-glycine (GG) motif in SUMO and the ε-amino group of a lysine residue in the target protein. A SUMO-specific protease generates a mature SUMO by cleaving C-terminal amino acids immediately following the double-glycine motif in precursor SUMO molecules (Bayer et al. 1998; Toshiká et al. 1999; Nishida et al. 2001). The conjugating system is an ATP-dependent enzymatic cascade that takes place in three steps (E1, E2, and E3). In the first step, SUMO is activated to form a thiolester linkage with the cysteine residue of the SUMO-activating enzyme (SAE) (E1). After activation, SUMO is transferred to the active-site cysteine of the SUMO-conjugating enzyme (SCE), E2 (Ubc9), forming a SUMO-Ubc9 thiolester intermediate (Desterro et al. 1997; Johnson and Blobel 1997; Schwarz et al. 1999; Sampson et al. 2001). For some target proteins, such as Ran GTPase-activating protein 1 (RanGAP1), SUMO can be transferred directly from E2 to the substrate (Matunis et al. 1996). However, in most cases, a specific SUMO ligase (E3) is required for efficient and proper transfer of SUMO from E2 to a target protein (Hochstrasser 2001). In mammalian cells, RWD-containing SUMOylation enhancer (RSUME) has been shown to interact with Ubc9 and enhances SUMO-1, -2, and -3 conjugation (Carbia-Nagashima et al. 2007). For deconjugation, a specific protease/hydrolase/isopeptidase is required to cleave the isopeptide bond between SUMO and its substrate (Melchior et al. 2003). In yeast, ubiquitin-like protease 1 (Ulp1) catalyzes both SUMO maturation and SUMO deconjugation (Li and Hochstrasser 1999).

Numerous proteins have been identified as SUMO target proteins since the discovery of SUMO in 1996, including the important regulatory proteins c-Jun, p53, PCNA, histone, and histone deacetylase (Seeler and Dejean 2003; Kerscher et al. 2006). Target proteins generally contain a consensus motif, ψKXE, where ψ represents a large hydrophobic amino acid, X is any residue, and E (glutamine) can be substituted by D (aspartic acid) (Sterndorf et al. 1999; Bernier-Villamor et al. 2002). This motif is sufficient for SUMOylation in vitro; however, for in vivo SUMOylation a nuclear localization signal is often required and interactions beyond those between the ψKXE motif and Ubc9, a SUMO conjugase, are likely to be critical for substrate selection (Kurtzman and Schechter 2001). In addition to ψKXE, several other sequence motifs have been found to be sites for SUMO attachment. These include TKXE, TKED, AKCP, VKYC, and VKFT (Johnson 2004). The requirement of both the nuclear localization sequence and SUMO consensus sequences can be used to search for putative target proteins in the Chlamydomonas reinhardtii genome. However, we have found that those short, unspecific motifs alone do not support in vivo SUMOylation. Fusion of putative target sequences to Ubc9 has been shown to aid in vivo detection of putative SUMOylation target proteins (Jakobs et al. 2007).

In plants, there is evidence that SUMOylation plays an important role in responding to stress and pathogens (Miura et al. 2007). The tomato SUMO homolog, LeSUMO, was shown in a yeast two-hybrid assay to interact with ethylene-inducing xylanase from the fungus Trichoderma viride, a strong elicitor of the rapid defense response in tomato. Moreover, the expression of LeSUMO in transgenic tobacco plants suppressed the induction of the defense response by ethylene-inducing xylanase, indicating that LeSUMO is likely to be a repressor in the plant defense pathway (Hanania et al. 1999). In A. thaliana, Western blot analysis of SUMO1/2 showed a significant increase of SUMO1/2 conjugates after exposure of seedlings to several stress conditions, such as heat shock, H2O2, ethanol, and the amino acid analog canavanine (Kurepa et al. 2003). In transgenic A. thaliana plants, overexpression of SUMO1/2 caused increased global SUMOylation levels, attenuated the abscisic acid-mediated growth inhibition, and induced the expression of abscisic acid and stress-responsive genes, such as RD29A (Lois et al. 2003). Dominant-negative A. thaliana mutants of the SUMO-conjugating enzyme ESD4 yield plants that are smaller and show delayed flowering (Reeves et al. 2002; Murtas et al. 2003). A mutation in a putative SUMOylation attachment site of long after far red light 1 (LAF1), a transcriptional activator for phytochrome A signaling, alters LAF1 accumulation in the nucleus (Ballesteros et al. 2001). A. thaliana SUMO E3 ligase (SIZ1) gene mutants show altered innate immunity (Lee et al. 2007) and an increased susceptibility to drought stress (Miura et al. 2007).

Although extensive proteomic analyses of SUMOylated proteins have been conducted in S. cerevisiae and mammalian cells (Denison et al. 2004; Rosas-Acosta et al. 2005), the sumoylation systems of other unicellular organisms, including C. reinhardtii, have not been in-
SUMOylation in *C. reinhardtii*

for Biotechnology Information using BLAST searches (Altshul et al. 1997). We retained database sequences annotated as SUMO proteins with significant similarity (e ≤ 10^-25) to the query. The final reference set included SUMO proteins from tomato, *S. ceroisia*, *S. pombe*, *Schistosoma mansoni* (Cabral et al. 2008), and other species.

Direct TBLASTN (Altshul et al. 1997) searches against genomes produced a high number, but only marginally significant, of hits due to missing certain short, primarily 5'-end exons. Accuracy was improved by selecting the 200,000-bp neighborhoods centered around each of the TBLASTN hits and performing genewiseDB searches (Birney et al. 2004) against these neighborhoods. GenewiseDB applies hidden Markov models (HMMs) for the exon/intron boundary predictions and for the assessment of the similarity to the query sequence. Narrowing the search space not only decreased the heavy computational load of the brute-force genewiseDB searches against the whole R4 genome but also resulted in more realistic gene predictions. In whole-genome searches, genewiseDB merged exons >0.5 million bp apart, an approach necessary for sizeable mammalian introns but not for the considerably shorter introns of the compact *C. reinhardtii* genome. We also performed estwiseDB searches (Birney et al. 2004) against the compact EST assemblies, where no such measures were necessary.

It is remarkable that the only apparent extensive modular feature of the SUMO architecture is moderately similar to the ubiquitin domain as indicated by HMMs from the PFAM (Finn et al. 2008) and the SMART (Schultz et al. 1998) protein domain databases. Unfortunately, the ubiquitin domain also is shared with a number of SUMO proteases, SUMO-activating enzymes, and other ubiquitin-related proteins that are not SUMOs. Also, the ubiquitin model missed *bona fide* *C. reinhardtii* SUMOs. This may explain why searches querying either the ubiquitin domain HMM or individual reference sequences produced a considerable number of hits, many of them false positives. This motivated our quest for more selective and sensitive HMMs. We selected well-characterized SUMO proteins (Figure 1) from the reference set, created their multiple alignment, and trained and calibrated a HMM using the HMMER package (Eddy 1998). This specific HMM query produced highly significant hits to the *C. reinhardtii* SUMO proteins.

The close sequence similarity of SUMO and other, functionally divergent ubiquitin-related proteins mandated conservative analyses. Therefore we eliminated a number of ubiquitin-related but not SUMO proteins. Such cases may be identified when the most similar sequences to a candidate are not annotated as SUMOs. We also eliminated proteins with less significant similarity that lacked the canonical glycine-glycine/asparagine cleavage site motif in the vicinity of the C-terminus. We also discarded a number of pseudogenes produced by gene duplication events. In these pseudogenes, the loss of function or the lack of transcription was indicated by in-frame stop codons, unusual codon usage, or overly divergent ubiquitin domains.

Scripts were written in the PERL programming language or MATLAB (MathWorks, Nantucket, MA). All computations were performed under the LINUX CentOS Operation System on Intel Xeon 64-bit processors. BLAST, genewiseDB, and estwiseDB searches, *augustus* predictions, and EST mappings were processed on a compute farm of 80 nodes under the Portable Batch System (PBS-PRO) job-scheduling software. Multiple alignments were created by the T-Coffee package (Notredame et al. 2000) and displayed by the Jalview Java Alignment Viewer (Clamp et al. 2004). Similarity trees were constructed by the first-order algorithm of the neighbor-joining method (Gascuel 1997). Nuclear and subnuclear
localization was predicted by the method of LEI and DAII (2005). Three-dimensional structure predictions were performed by using the Swiss-Model comparative protein modeling server (http://swissmodel.expasy.org/swissmod/SWISS-MODEL.html).

Chlamydomonas strains, growth conditions, and stress treatments: C. reinhardtii wall-less, wild-type strain CC-503 and walled, wild-type strain CC-125 were originally obtained from the Chlamydomonas Genetics Center at Duke University (Durham, NC). They were maintained on Tris-acetate phosphate (TAP) plates containing 1.2% agar (HARRIS 1989) at 25°C under constant light (60 μE⋅m⁻²⋅sec⁻¹). For RNA or protein isolation, cells were inoculated into liquid TAP media (HARRIS 1989), unless indicated otherwise, and allowed to grow under continuous light at 25°C on a rotary shaker at 135 rpm to a density of ~0.5–1 × 10⁷ cells/ml. For heat-shock experiments, midlog-phase cells were transferred to incubators prewarmed to 37°C or 42°C and grown for the indicated time. For osmotic stress treatments, sorbitol or sodium chloride was added to the reaction mixture that contained the vendor’s master mix, 0.25 μM of total RNA in a Plexor Two-Step qRT–PCR system (Promega) both with and without reverse transcriptase and subsequently diluted 1:20 in 1 mM MOPS, 0.1 mM EDTA. Quantitative PCR was carried out in a 25-μl mixture that contained the vendor’s master mix; 0.25 μM of each primer (Biosearch Technologies, Novato, CA), and 5 μl cDNA. The primer sets were separately tested for efficiency. The reaction conditions for the ABI 7500 were 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec, 65°C for 30 sec, with dissociation conditions of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Primer sets used for real-time RT–PCR reactions were as follows: CrSUMO-like89A, 5′-GTGGAGAACGTTT-3′ and 5′-AATCAACAGCTTGGCAGA GTC-3′, 89% efficiency; CrSUMO-like90, 5′-CAGGCCCTCCTTT GTTGCCAGTAG-3′ and 5′-CAACATCATTATCAAGGACAGG GTG-3′, 90% efficiency; CrSUMO96, 5′-AGTGGCATCACCACA TTAACCCGGC-3′ and 5′-CTCACCGGTCATTCAGTGATTG-3′, 96% efficiency; CrSUMO97, 5′-GGAGCCCCCTCCTTT GGTGAGTTAG-3′ and 5′-CAACATCATTATCAAGGACAGG GTG-3′, 90% efficiency; CrSUMO98, 5′-AGTCTCTCAACTCAT GAAACCC-3′ and 5′-GGATACCGTGCCATGGTATTTG-3′, 96% efficiency; CrSUMO99, 5′-GGCTGCTTGCCGCAGCAACACAT TGAAACCC-3′ and 5′-GGATCCGTTAGGATAGGTTGG-3′, 96% efficiency; and CIA5, 5′-GGCTGCTTGCCGCAGCAACACAT TGAAACCC-3′ and 5′-GGATCCGTTAGGATAGGTTGG-3′, 96% efficiency; and CIA5, 5′-GGCTGCTTGCCGCAGCAACACAT TGAAACCC-3′ and 5′-GGATCCGTTAGGATAGGTTGG-3′, 96% efficiency; and CIA5, 5′-GGCTGCTTGCCGCAGCAACACAT TGAAACCC-3′ and 5′-GGATCCGTTAGGATAGGTTGG-3′, 96% efficiency; and CIA5, 5′-GGCTGCTTGCCGCAGCAACACAT TGAAACCC-3′ and 5′-GGATCCGTTAGGATAGGTTGG-3′, 96% efficiency. Quantitative PCR on minus reverse transcriptase reactions did not show signal, confirming the absence of detectable DNA in the input total RNA. The 2⁻SA₅ method (LIJAK and SCHMITTGEN 2001) was used to compare relative transcript abundance in the 25°C and 42°C samples normalized to an endogenous reference gene, CIA5 (XIAN et al. 2001). Efficiency-corrected ΔCt values of the different CrSUMO mRNAs in each cell sample, normalized to CIA5 mRNA were compared to the lowest abundant transcript, CrSUMO89A mRNA, which was set at 1.0 for comparison purposes. Levels of CIA5 mRNA did not change under the conditions tested in this work.

Cloning, total RNA isolation, RT–PCR, and plasmid construction: Total RNA was isolated as described before (XIAN et al. 2001), and cDNA coding regions for CrSUMO96, CrSUMO148, and CrUBCE2-1 were reverse transcribed and amplified by using the following primer sets: CrSUMO96-forward, 5′-TCCGACTCATCATGGCCAGACAGGAG GCTAG-3′ (Xhol site underlined); CrSUMO96-reverse, 5′-CCTG ACTGGGCGGAGGCTGCAGCGCGGCGAGCT-3′ (Xhol site underlined); CrSUMO148-forward, 5′-ACCATGCGCAGGTTAAG GTGAGGGGATCA-3′ (Nol site underlined); CrSUMO148-reverse, 5′-CCTGGAGGCTGTTCGGTGGATGCACGACACAG-3′ (Nol site underlined); CrSUMO-like89A-reverse, 5′-CCTGGAGGCTGTTCGGTGGATGCACGACACAG-3′ (Nol site underlined); CrSUMO-like89A-forward, 5′-CCTGGAGGCTGTTCGGTGGATGCACGACACAG-3′ (Nol site underlined).

Antisera, immunoblots, and immunoprecipitation: Polyclonal antibodies were raised against purified His-CrSUMO96, His-CrSUMO148, and His-CrSUMO-like89A (Cocalico Biologicals, Reamstown, PA). Antibody affinity purification was performed according to ERMOLI et al. (2003). Arabidopsis anti-SUMO1 antibody was a gift from Richard Vierstra (University of Wisconsin, Madison, WI), and the anti-histidine tag antibody was purchased from CloneTech Laboratories (Mountain View, CA).

For protein electrophoresis and immunoblots, exponentially growing cells were harvested and resuspended in a density of ~0.5–1 × 10⁹/ml with Tris-buffered saline (TBS). In experiments detecting SUMO-conjugated proteins, protease inhibitor cocktail (Sigma) and 2 mM N-ethylmaleimide (NEM) were added to TBS. Resuspended cells were mixed with an equal volume of 2× SDS sample buffer, boiled, and resolved by SDS–PAGE (LAEMMLI 1970), using 10% polyacrylamide (w/v) separation gels and 5% polyacrylamide (w/v) stacking gels. Proteins were transferred onto nitrocellulose membranes (Amersham Biosciences) with a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories). The membranes were blocked in TBS containing 5% milk powder and probed with antibodies diluted in blocking buffer. Detection employed horseradish peroxidase (HRP)-labeled donkey anti-rabbit immunoglobulins (Amersham Biosciences) in conjunction with Super Signal chemiluminescence (Pierce, Rockford, IL).
and X-Omat autoradiographic film (Eastman Kodak, Rochester, NY).

For two-dimensional PAGE analysis of proteins from control cells grown at 25°C and from similar cell cultures exposed to a 42°C heat-shock treatment for 1 hr, 20 ml of cells at ~3 × 10^6 cells/ml were collected by centrifugation and extracted with acetone and phenol (Hajdusek et al. 2005). A total of 400 μg of the resulting proteins from each sample were separated by isoelectric focusing using a 4–20% gradient SDS–polyacrylamide gel with a 4% stacking gel. After transfer of the proteins to a nitrocellulose membrane, the blot was incubated in a polyacrylamide gel with a 4% stacking gel. After transfer of the proteins separated by isoelectric focusing using an 11-cm ReadyStrip IPG strip, pH 3–10 (Bio-Rad). Proteins separated by isoelectric focusing were detected using a SuperSignal West Pico chemiluminescent substrate (Pierce).

For immunoprecipitation experiments, exponentially growing cells were harvested and washed with TBS and then resuspended to a density of ~0.5–1 × 10^7/ml with lysis buffer (TBS, 1% (v/v) Tween-20, and protease inhibitor cocktail (Sigma)). Cells were broken with a sonication pulse pattern (pulse on 1 sec, pulse off 1 sec, 1-min cycle repeated six times), using a tapered microtip and a VCX600 ultrasonic processor (Sonic & Materials, Danbury, CT). In some cases, sonicated cell lysate was heated to 90°C for 30 min. Clarified cell lysate was mixed with 10 μg purified anti-CrSUMO96 polyclonal antibody, 20 μl 25% protein A agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and protease inhibitor cocktail (Sigma), diluted with TBS to a final volume of 1 ml. The reaction was incubated at 4°C for 2 hr, and the immunocomplexes were washed three times with lysis buffer and eluted with 2 × SDS sample buffer. The resulting proteins were resolved by SDS–PAGE, detected by silver staining or transferred to nitrocellulose membranes, and detected with anti-CrSUMO96 antibody (Bio-Rad Laboratories).

Mass spectrometric peptide sequencing of CrSUMO96: Proteins purified by immunoprecipitation were excised from a silver-stained gel. Each protein band was cut into ~1-mm³ pieces and desalted. After destaining, gel pieces were dehydrated with 100% acetonitrile and dried under vacuum for 15 min. The gel pieces were rehydrated in a solution of 40 mM NH₄HCO₃, 10% acetonitrile, and 20 μg/ml trypsin and incubated at 37°C overnight. The peptides were subjected to HPLC/mass spectrometry (MS)/MS analysis performed by the mass spectrometry core facility in the Redox Biology Center of the University of Nebraska (Lincoln, NE).

Immunofluorescence localization assay: For immunofluorescence experiments, cells were grown to a density of ~2–5 × 10^6 cells/ml and fixed by addition of 10 μl of formaldehyde (Fisher Scientific Chemicals, Fairlawn, NJ) and incubated at room temperature for 3 min. Cells were then collected by centrifugation (3 min at 3000 × g), washed with phosphate-buffered saline three times, resuspended in cold methanol, and kept overnight at ~20°C. An aliquot of 100 μl of cells at a density of ~2–5 × 10^6 cells/ml was spotted onto a polycarbonate coverslip (poly-prep, Sigma) and allowed to stand for 10 min. Fixation and extraction of pigments were conducted by plunging the coverslip into a Coplin jar containing methanol at ~20°C and incubating the slide in the cold methanol for 5 min. This procedure was then repeated. The CrSUMO96 primary antibody and the cyanine-5-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted 1:100 and 1:60, respectively. Sytox green (Molecular Probes, Eugene, OR) in PBS was used for DNA staining at 0.25-μM final concentration. Images were photographed using an Olympus Fluorview FV500 confocal laser scanning system with an Olympus BX60 fluorescence microscope (Olympus America, Melville, NY). This immunofluorescence procedure was based on a protocol provided by Susan Dutcher (Washington University, St. Louis).

In vivo E2 ligase assay: Plasmids pTE1E2S1 and pET28-RanGAP1-C2 were obtained from Hisato Saitoh (Kumamoto University, Japan). UBC9 (E2) in the original plasmid pTE1E2S1 was eliminated by removing the small Ndel fragment and circularizing the plasmid, designated as pTE1E2S1ΔE2. Plasmid cotransformation was conducted as follows: 1 μg of each of the pTE1E2S1ΔE2 and pET28-RanGAP1-C2, with or without 1 μg pGEX1TI-CrUbcE2B-1, was mixed with 100 μl BL21 competent cells. After incubation for 30 min on ice followed by a heat-shock treatment, 1 ml of Luria–Bertani (LB) media was added. The bacteria were incubated at 37°C for 2 hr and plated on LB plates containing 50 μg/ml chloramphenicol and 25 μg/ml kanamycin, with or without 100 μg/ml ampicillin. Colonies were picked randomly and inoculated in LB media containing appropriate antibiotics. After IPTG induction at 37°C for 4 hr, total soluble protein was separated by SDS–PAGE. The expression of RanGAP1-C2 was detected by immunoblot analysis with HRP-conjugated anti-6X His antibody (Clontech Laboratories), and the expression of CrUbcE2_1 was detected with anti-GST antibody (Santa Cruz Biotechnology).

RESULTS

SUMO and SUMO-like proteins are encoded by at least six genes in C. reinhardtii: C. reinhardtii SUMO genes and proteins in V3.1 of the genome determined by the Department of Energy Joint Genome Institute (JGI) were complemented by our gene/protein models for V4 of the genome. We performed the optimal mapping of the ESTs we assembled over the V4 genome by the GMAP tool (Wu and Watanabe 2005) and predicted genes using the augustus algorithm (Stanke and Waack 2003). Databases were queried by protein and translated BLAST, position-specific iterative BLAST (Altschul et al. 1997), HMMER (Eddy 1998), estwise, and TBLASTN-directed genewise (see Computationalbiology methods) (Birney et al. 2004) searches. Sequence reference sets and SUMO HMMs were developed as described in Computational biology methods. Initially, four SUMO homologs were identified and named CrSUMO96, CrSUMO148, and CrSUMO-like89A and CrSUMO-like89B, respectively, on the basis of the length of their predicted amino acid sequences (Table 1). They share 68, 54, 56, and 61% amino acid sequence similarity with the closest human SUMO homologs and 64, 34, 56, and 56% similarity with A. thaliana SUMO1, respectively (Figure 1, Table 1). In version 4 of the JGI database of the Chlamydomonas genome, a fifth SUMO homolog was discovered. CrSUMO96, CrSUMO-like90, was discovered. The amino acid
sequences of the three CrSUMO proteins and the C-terminal amino acid sequences of the three CrSUMO-like proteins are presented in Figure 1 along with amino acid sequences of SUMOs from C. reinhardtii, A. thaliana, S. cerevisiae, and H. sapiens. A high degree of similarity between the various SUMO sequences is observed (Table 1 and Figure 1).

The C-terminal segment, CSCALE, in CrSUMO96 and the GVSA segment in CrSUMO97 precursor proteins are cleaved to bring the canonical GG motif into the C-terminal position. This double-G motif functions as the attachment site for the SUMO-activating enzyme, E1, and, ultimately, it reacts with an ε-amino group of a lysine residue within a protein targeted for SUMOylation. CrSUMO148 presents a unique SUMO structure. It possesses five separate double GG potential cleavage sites within its long C-terminal domain. Three of the double-G motifs are part of a perfect IDAFVQEGG repeat (Figure 1).

The three CrSUMO-like proteins are distinctly different from the three CrSUMO proteins with regard to a number of features. First, they are distinguished by the lack of a double-G motif at the C termini. Instead, they possess nonprocessed glycine-asparagine C termini (i.e., the mRNAs for these proteins contain glycine and asparagine codons immediately upstream of a termination codon, producing “GN” C termini). Second, these proteins possess unusual proline inserts/substitutions near the C termini of the proteins (positions 92 and 104 for CrSUMO-like90, position 92 for CrSUMO-like89A, and position 104 for CrSUMO-like89B in Figure 1). Very close proximity of the three closely related CrSUMO-like genes on chromosome 17 (Table 2) suggests they may have arisen from two gene duplication events. Finally, the presence of long upstream reading frames for the genes encoding the three CrSUMO-like proteins (http://genome.jgi-psf.org/Chlr3/Chlr3.home.html) is consistent with the potential existence of these proteins as fusions of the SUMO-like proteins to the C termini of three separate and possibly unrelated proteins.

Protein identification numbers and other characteristics of each of the SUMO and SUMO-like proteins are provided in Tables 1 and 2. A similarity tree representing the amino acid sequence distance among SUMOs in different species was constructed, using the first-order algorithm of the neighbor-joining method (Gascuel 1997) (supplemental Figure 1).

CrSUMO96 is encoded by a gene containing five exons. A ψKXE consensus motif, VKTE, was found at the N terminus of the polypeptide, indicating the possibility of poly-SUMOylation. Cleavage by an Ulp hydrolyase/isopeptidase after the tandem glycine residues would produce a mature SUMO of 90 amino acids in length. The 3-D structure of CrSUMO96 was predicted using the Swiss-Model automated comparative protein-modeling server. The predicted 3-D structure is very similar to that of the human SUMO-1 except for a slight difference at the N terminus (Figure 2). The highly similar CrSUMO97 is encoded by a gene with eight exons. As with CrSUMO96, CrSUMO97 has a C-terminal GG motif followed by 4 amino acids in the immature precursor and contains an N-terminal ψKXE SUMOylation site motif.

The CrSUMO148 gene contains six exons with a predicted reading frame of 148 amino acids. The CrSUMO148 protein also contains a ψKXE consensus motif, VKAE, at the N terminus. Cleavage after the first double-glycine repeat would produce a mature protein of 83 amino acids in length. Cleavage after the second, third, fourth, and
fifth double-glycine repeats would produce mature proteins of 93, 103, 113, and 135 amino acids, respectively.

Real-time RT–PCR detected mRNAs of CrSUMO-like89A, CrSUMO-like90, CrSUMO96, and CrSUMO148 (Figure 3). CrSUMO97 mRNA was not detected in these experiments, even with input RNA of 1 μg. CrSUMO-like89B mRNA was not investigated. There was no detectable difference in the respective CrSUMO mRNA abundance between samples from cells that were shifted from 25°C to 42°C for 1 hr, and 2^-ΔΔCt values for those conditions resulted in values close to 1.0. However, some CrSUMO transcripts are more highly expressed than others. CrSUMO96 mRNA was dramatically more abundant when compared to CrSUMO-like89A expression levels, and CrSUMO-like90 and CrSUMO148 mRNAs consistently showed slightly more abundance than CrSUMO89A mRNA (Figure 3). Samples from walled CC-125 cells and wall-less CC-503 cells revealed similar expression patterns (data not shown).

The cDNA coding regions for CrSUMO96 and CrSUMO148 were amplified by RT–PCR and that for CrSUMO-like89A by PCR. All three were cloned into the pET28 expression vector for protein expression in E. coli. Attachment of six histidines (6× His tag) to each protein allowed purification of C. reinhardtii SUMO proteins (Figure 4, lanes 2–4). These polypeptides were recognized by the anti-6× His antibody (Figure 4, lanes 5–7). Polyclonal antibodies were generated in rabbits against bacterially expressed and purified antigens. In immunoblot analysis, the anti-CrSUMO96 antibody recognized recombinant (r)CrSUMO96 and showed a slight cross-reaction with rCrSUMO148 (Figure 4, lanes 11–13). Similarly, the anti-CrSUMO148 antibody recognized rCrSUMO148 and showed cross-reaction with rCrSUMO96 (Figure 4, lanes 14–16). Because of the high degree of similarity between CrSUMO96 and CrSUMO97, we assume, but have not demonstrated, that the polyclonal antibodies raised against rCrSUMO96 likely will also recognize CrSUMO97—if, indeed, CrSUMO97 is produced (Figure 3). Interestingly, the anti-CrSUMO-like89A antibody detected rCrSUMO-like89A in an immunoblot but showed much weaker affinity for rCrSUMO-like89A than for rCrSUMO96 and rCrSUMO148 (Figure 4, lanes 17–19). Arabidopsis anti-SUMO1 antibody detected only rCrSUMO96 on immunoblots (Figure 4, lanes 8–10).

**Purification and mass spectrometry analysis of CrSUMO96 in C. reinhardtii:** The decision to focus on CrSUMO96 for further study was made because of prior knowledge that the double-glycine motif was critical for the isopeptide bond formation when the activated SUMO conjugates to a target protein (Johnson 2004; Kerscher et al. 2006). The CrSUMO148 predicted coding region contains five double-glycine motifs, while the CrSUMO-like proteins contain none. Moreover, of the SUMO cDNAs we have cloned and expressed in E. coli, rCrSUMO96 was also the only C. reinhardtii SUMO
homolog to be recognized by Arabidopsis anti-SUMO1 
antibody.

Because A. thaliana free SUMO1 and SUMO3 were 
reported to be resistant to treatment at 90°C for 30 min 
(Kurepa et al. 2003), we tested to determine if this 
feature of SUMO could be used for enrichment of en-
dogenous free SUMO from C. reinhardtii. After a 
cells extract was heated to 90°C for 30 min and 
clarified by centrifugation, the endogenous free SUMO 
corresponding to the size of CrSUMO96 remained 
soluble and was detected by immunoblot analysis with 
the anti-CrSUMO96 antibody (Figure 5). These experi-
ments also showed that approximately three times the 
amount of heated cell extract was tripled (Figure 5, lanes 4 and 7).

To confirm the identity of CrSUMO96, immunopre-
cipitation experiments were performed using anti-
CrSUMO96 antibody. The immunoprecipitated fraction 
was shown to be enriched with CrSUMO96 by immuno-
blot (with anti-CrSUMO96 antibody; supplemental Fig-
ure 2, left) and by silver stain (supplemental Figure 2, 
right). A similar pattern was seen in that the free 
CrSUMO96 increased respective to the amount of heated 
cell extract added to the reaction. The endogenous free 
SUMO purified by immunoprecipitation was recovered 
from the silver-stained gel and analyzed by mass spectrom-
etry. The presence of two peptide fragments containing 
amino acid sequences identical to predicted fragments 
from CrSUMO96 protease digestion confirmed the iden-
tity of the protein (supplemental Figure 3).

Detection of SUMO-conjugated proteins by immu-
noblot analysis: To detect SUMO-conjugated proteins 
in C. reinhardtii, immunoblot analysis was performed 
using anti-CrSUMO96 antibodies (Figure 4). In addi-
tion to free CrSUMO96 migrating at ~15 kDa, a large 
number of SUMO-conjugated proteins were detected, 
suggesting that all the enzymes needed in the SUMO-
conjugating system are present and functional in C. 
reinhardtii. NEM, an inhibitor of SUMO-specific isopep-
tidases (Li and Hochstrasser 1999), was added to 
protect SUMO-conjugated proteins from desumoyla-
tion. When NEM was added to the extraction buffer, 
more SUMO-conjugated proteins and less free SUMO 
were detected by the antibody (Figure 6, lanes 3 and 4). 
However, a few proteins still exist, such as the 26-kDa 
protein (⁎ in Figure 6), that did not change intensity 
after NEM was added, indicating that antibodies to 
these proteins may also be present in our anti-
CrSUMO96 antisera.

Subcellular localization of CrSUMO96 and its 
conjugated proteins: The subcellular localization of 
CrSUMO96 and its conjugated proteins was detected 
by immunofluorescence (Figure 7). Anti-CrSUMO96 
antibody was recognized by a secondary cyanine-5-
conjugated goat anti-rabbit antibody that emits a far-
red fluorescence signal under illumination with light of 
630 nm. Sytox green was employed to stain DNA and 
allow detection of the nucleus during confocal micros-
copy. As shown in Figure 7, CrSUMO96 and its protein 
conjugates are localized primarily, if not exclusively, to

![Figure 2.—Predicted 3-D structure of human SUMO1 and CrSUMO96.](image-url)
the nucleus of the cell. This observation is in agreement with previous observations that most of the SUMO conjugates in mammalian cells are found in the nucleus (Johnson 2004; Kerscher et al. 2006).

SUMO-conjugating enzyme (E2) from C. reinhardtii: In contrast to most ubiquitin-conjugating systems, there is only one SUMO E2 enzyme in yeast, mammals, and A. thaliana. The number of CrUBCE2 genes present in the C. reinhardtii genome is uncertain. As many as 12 homologs can be discerned. Using HMM analysis and conservative selection parameters, four predicted CrUbcE2 molecules designated CrUbcE2_1, CrUbcE2_2, CrUbcE2_3, and CrUbcE2_4 were selected as most likely to function as SUMO-conjugating enzymes. All of the gene sequences predict an open reading frame that contains a conserved ubiquitin-conjugating enzyme catalytic (UBCC) domain (IPR000608). However, it is difficult to distinguish SUMO E2 from ubiquitin E2’s by amino acid sequence alone. This is exemplified by the fact that the degree of sequence similarity between SUMO E2 and the various ubiquitin E2’s in yeast is comparable with that between the various yeast ubiquitin E2’s (Johnson et al. 1997).

It has been reported that SUMO E2 conjugases have a much more positive net charge at neutral pH than ubiquitin E2’s (Johnson and Blobel 1997). The calculated isoelectric points (pI) for each of the four selected CrUbcE2 candidates are listed in supplemental Figure 5A. The potential C. reinhardtii E2 that ranked highest in similarity to authentic Ubc9-like E2 conjugases, CrUbcE2_1 (XP_001694849), was amplified by RT–PCR and used for additional studies.

The predicted amino acid sequence of CrUbcE2_1, as derived from the sequenced RT–PCR product, is shown in supplemental Figure 4. Alignment of CrUbcE2_1 and the amino acid sequence of other CrUbcE2’s with Ubc E2’s from other species (Figure 8) shows a marked similarity between the putative CrUbcE2’s and the E2-conjugating enzymes from other eukaryotes. Calculation of the isoelectric point of CrUbc2EB_1 confirmed an alkaline pI of 8.81 for this molecule (supplemental Figure 5A). A similarity tree representing distances among E2 homologs is depicted in supplemental Figure 4B.

An in vivo SUMOylation experiment was conducted using an in vivo E. coli system established by the laboratory of Hisato Saitoh (Uchimura et al. 2004a,b) to

---

**Figure 3.**—Relative abundance of C. reinhardtii CrSUMO transcripts. Expression of the candidate SUMO genes was analyzed by quantitative real-time reverse transcription–PCR for expression in CC-503 cells at 25°C and 42°C. Each 25-μl reaction mixture contained cDNA equivalent to 50 ng of total input mRNA. Relative abundance was calculated with efficiency-corrected ΔCt values. Each data point is the average of an experimental triplicate and represents an individual trial.

**Figure 4.**—Immunodetection of C. reinhardtii CrSUMO96, CrSUMO148, and CrSUMO-like89A with anti-CrSUMOs and anti-AtSUMO-1 antibodies. Overexpression and purification of 6× His-tagged CrSUMOs from E. coli, antiserum production, and immunoblot detection are shown. CrSUMO96 overexpressed in E. coli and affinity purified on a 6× His column (lanes 2, 5, 8, 11, 14, and 17), overexpressed and purified CrSUMO148 (lanes 3, 6, 9, 12, 15, and 18), and overexpressed and purified CrSUMO-like89A (lanes 4, 7, 10, 13, 16 and 19) were separated on 12% SDS–PAGE and stained with Coomassie blue (lanes 1–4) or detected after immunoblotting using anti-6× His antibody (lanes 5–7), anti-AtSUMO1 antiserum (lanes 8–10), anti-CrSUMO96 antiserum (lanes 11–13), anti-CrSUMO148 antiserum (lanes 14–16), and anti-CrSUMO-like89A antiserum (lanes 17–19).
determine if CrUbcE2_1, the C. reinhardtii SUMO E2 conjugase with the highest similarity to authentic human and yeast E2 conjugases as determined by HMM analysis (supplemental Figure 4A), was a potential SUMO conjugase. This system utilizes E. coli BL21 cells transformed with pT-E1E2S1, a plasmid that contains genetically engineered versions of genes encoding mouse E1-activating enzyme, Xenopus laevis E2-conjugating enzyme, and human SUMO1. Transformation with an additional plasmid, pRanGAP1-C2 leads to production of a histidine-tagged RanGAP1 C-terminal region (RanGAP1-C2), a well-known SUMO target protein. Incubation of BL21 cells carrying the pRanGAP1-C2 plasmid alone, as expected, did not produce SUMOylated RanGAP1-C2 because the SUMO-conjugating system is absent in E. coli (Figure 9, top, lane 1). However, when BL21 cells carry both plasmids (pT-E1E2S1 and pRanGAP1-C2), RanGAP-C2 is produced and a SUMOylated version of this target protein is synthesized in vivo (Figure 9, lane 4, band A). Expression of the putative E2 CrUbcE2_1-conjugating enzyme in transformed BL21 cells was confirmed by immunoblot detection of the GST-tagged C. reinhardtii E2 conjugase (Figure 9, bottom, lane 4, band C). Compared with the vertebrate Ubc9, the heterologous C. reinhardtii SUMO-conjugating enzyme worked with a lower efficiency in this in vivo E. coli sumoylation system (Figure 9, lane 2 vs. lane 4, band A). This is not surprising, considering the multiple interactions required between the C. reinhardtii SUMO E2 and the three heterologous mammalian proteins (E1, SUMO, and the target protein RanGAP1-C2) for successful SUMOylation to take place. Because of its apparent successful function in the in vivo SUMO E2 conjugase assay, CrUbcE2_1 becomes a prime candidate as an authentic C. reinhardtii SUMO-conjugating enzyme.

Stress-induced accumulation of SUMO-conjugated proteins: Because it has been reported that SUMO conjugation is part of the stress response in animals and plants (Hong et al. 2001; Kurepa et al. 2003), immunoblot experiments were performed to detect SUMO-conjugated proteins isolated from C. reinhardtii cells grown in various stress and nonstress conditions (Figure 10). When C. reinhardtii cells were shifted from optimal growth temperature of 25° to 37°, an increase in SUMO-conjugated proteins was detected with anti-CrSUMO96 antibodies (Figure 10A, lanes 2 and 3). Moreover, when the cells were shifted from 25° to 42°, a marked increase...
in SUMO-conjugated proteins was detected in the molecular size range of \( \geq 60 \) kDa. Interestingly, at this high temperature no free CrSUMO96 was detected, indicating that most free CrSUMO96 was incorporated into SUMO target proteins under this stress condition (Figure 10A, lanes 8 and 9). A more detailed examination of proteins from control and heat-shocked cells by 2-D PAGE analysis (supplemental Figure 6) demonstrates again that heat shock results in a marked decrease in the amount of nonconjugated CrSUMO’s (thick-walled red boxes). Concomitantly, there is a pronounced increase in the degree of SUMOylation of several proteins (green boxes), including the de novo appearance of SUMOylated forms of a number of additional proteins. Reaction of identical protein blots with preimmune serum demonstrates the specificity of the CrSUMO96 antiserum for free CrSUMO96 and CrSUMO96 conjugated to larger proteins. Only weak detection of a few non-SUMO-related proteins was observed using the preimmune serum (supplemental Figure 6).

Osmotic stress also induced the accumulation of SUMO-conjugated proteins (Figure 10B). When 200 mM sorbitol or 100 mM NaCl was added to the cells, more SUMO-conjugated proteins were detected with the anti-CrSUMO96 antibody as compared with the cells

---

**Figure 7.**—*In situ* localization of CrSUMO96 and its conjugated proteins by immunofluorescence. Wild-type *C. reinhardtii* cells grown in TAP media were stained with Sytox Green (top left) or detected with anti-CrSUMO96 antibody, which is recognized by the red fluorescent cyanine-5-conjugated goat anti-rabbit antibody (top middle). Images under transmitted light and confocal images are also shown in the top right and the bottom.

**Figure 8.**—SUMO-conjugating enzyme E2 from *C. reinhardtii* amino acid sequence alignment of human, yeast, *A. thaliana*, and *C. reinhardtii* SUMO-conjugating enzyme (E2).
grown in normal media (Figure 10B, lane 2, 3, 5, 6, 8, and 9). It appears that some SUMO-conjugated proteins induced by the osmotic stress are distinct from those induced by heat shock, because heat shock induced the accumulation of SUMO-conjugated proteins with a molecular size of 60 kDa. In contrast, osmotic stress induced SUMOylation of larger proteins, as well as proteins 60 kDa (Figure 10, A (lanes 2, 3, 8, and 9) and B (lanes 2, 3, 8, and 9)).

DISCUSSION

SUMO and other ubiquitin-like proteins in C. reinhardtii: Our investigations have revealed six putative CrSUMO and CrSUMO-like proteins and several potential SUMO-conjugating enzymes (CrUbc9’s) in the unicellular, photosynthetic alga, C. reinhardtii. The six C. reinhardtii SUMO homologs have been designated as CrSUMO96, CrSUMO97, and CrSUMO148 and CrSUMO-like89A, CrSUMO-like89B, and CrSUMO-like90. They share higher sequence identity with human SUMO1 (Figure 1 and supplemental Figure 1). There are 15 proteins listed as ubiquitin-like proteins in the Cluster of EuKaryotic Orthologous Groups (KOG) Browser in the JGI C. reinhardtii database. Most contain a ubiquitin domain; however, only CrSUMO96, CrSUMO97, and CrSUMO148 and CrSUMO-like89A, CrSUMO-like89B, and CrSUMO-like90 have significant sequence similarity with known SUMOs in other species (Figure 1).

Of the six C. reinhardtii SUMO homologs, the closely related CrSUMO96 and CrSUMO97 contain a ψKXE consensus motif at their N termini. CrSUMO96 has a very similar predicted 3-D structure when compared with human SUMO1 (Figure 2). Moreover, CrSUMO96 is recognized by A. thaliana anti-SUMO1 antibody (Figure 4). Unlike any known SUMO proteins, CrSUMO148 contains five double-glycine motifs at the C terminus. The observations that all three CrSUMO-like proteins contain a GN dipeptide at their C termini instead of the canonical double-glycine motif (Figure 1) and the likely possibility that all three genes encoding the CrSUMO-like proteins contain potential large open reading frames upstream of the SUMO-like domain suggest the CrSUMO-like proteins may be related to a class of fusion proteins con-
taining highly conserved ubiquitin sequences at either the N-terminal or the C-terminal ends (Kerscher et al. 2006; see Kramer et al. 1995 for an extensive list of fusion proteins with ubiquitin-like terminal domains). These fusion proteins, thus, can bind to ubiquitin-binding domains (UBDs) and carry along their fusion partner to perform a particular function. Two examples of proteins with ubiquitin sequences at their C termini, both involved in pre-mRNA splicing, are SF3a120 (Kramer et al. 1995) and CePRP21 (Spikes et al. 1994). Whether the three CrSUMO-like proteins perform functions analogous to ubiquitin-containing fusion proteins remains to be determined.

Other components of the SUMO-conjugation system in C. reinhardtii: In addition to free GrSUMO96, proteins conjugated to GrSUMO96 also can be detected with anti-CrSUMO96 antibodies in immunoblot analyses of cell extracts (Figures 5, 6, and 10 and supplemental Figure 6). These observations strongly suggest that all enzymes needed for SUMO conjugation are present and functional in C. reinhardtii. We have demonstrated that XP_001694849 (CrUbcE2_1) is a prime candidate as an authentic SUMO-conjugating enzyme (SCE, E2) in C. reinhardtii (Figure 9). Predicted amino acid sequence of CrUbcE2_1 shares 43% identity and 56% similarity with yeast Ubc9p and 49% identity and 59% similarity with A. thaliana Ubc9 (Figure 6). Like SUMO E2’s of other species, CrUbcE2_1 has a basic isoelectric point (i.e., pI = 8.81) that might be necessary for it to interact with free SUMO and other components in the SUMO-conjugating system (Johnson 2004). The most compelling argument suggesting that XP_001694849 (CrUbcE2_1) encodes a bona fide SUMO E2 conjugase is our demonstration of SUMO E2 conjugase activity in an E. coli in vitro SUMOylation system (Figure 9).

SAE1: A putative C. reinhardtii SUMO-activating enzyme 1 (SAE1, XP_001690572) shares remarkable patterns with the yeast Aos1 (SAE1) (CAA97885) molecule. These patterns include an SGG and an RGG cleavage site at the C termini and a series of conserved charged residues as well as serines, threonines, and tyrosines both at the cores and at the C termini. It is intriguing whether the lack of similarity at the N terminus is a result of functional divergence. Overall, 40% of the residues are identical and 54% are similar between the two proteins.

SAE2: Two putative C. reinhardtii SUMO-activating enzyme 2 (SAE2) proteins (XP_001691317 and XP_001690945) align with a similarity of 67 and 80%, respectively, to the A. thaliana SAE2 (NP_973506) at three extensive but somewhat disjunct domains. Clearly, these domains are not related to the ubiquitin or the SUMO domains. The two proteins have somewhat different variants (KFPLCTLAETP, LPPLCPAPASP, and KDPSCPCASGVP) of the motif KXP/GTXXXP containing the cysteine in SAE2 that forms the thioester intermediate with SUMO in SAE2 molecules of other eukaryotes. We also note that XP_001690945, in spite of its original annotation, is more closely related to SAE2 than to SAE1 enzymes.

SUMO E3 ligase: Currently it is difficult to distinguish SUMO E3 ligases from those responsible for adding ubiquitin or other ubiquitin-like peptides, such as NEDD8, to their target proteins. There are 26 “ubiquitin-protein ligases” and 34 “E3 ubiquitin ligases” predicted in the KOG Browser of the C. reinhardtii database.

Ubiquitin, UFM1, and RUB: HMM and BLAST searches identified multiple potential ubiquitin genes and pseudogenes in C. reinhardtii. Nonetheless, a minimum of five ubiquitin proteins could be identified with a high level of confidence. They are ubiquitins XP_001694320 (242 residues), XP001702404 (244 residues), and XP_001147989 (77 residues, enigmatically 97% identical to a Pan troglodytes coiled-coil 99 protein), predicted protein 140045 (153 residues), and biubiquitin XP_001694608 (153 residues) (supplemental Figure 7). Each of the last two proteins is composed of two very similar internal duplicate segments. With the exception of XP_001694320, they all contain a predicted double-glycine motif near the C terminus of the peptide, and all have a PFAM ubiquitin domain in the predicted sequence. A protein sequence (XP_001696636) with 77% sequence similarity to human Ufm1 (Table 1) suggests that C. reinhardtii contains at least one representative of this rarer ubiquitin-related family of proteins. Each of C. reinhardtii proteins XP_001694608 (biubiquitin) and 1794 (both containing 153 residues) is >95% identical to other ubiquitin-related proteins in Populus trichocarpa, Tetrahymena thermophyla SB210, and other organisms. These two proteins are the closest homologs of the A. thaliana related to ubiquitin-1 protein (RUB1, NP_564379) and the XP_001694608 sequence is 94% identical to RUB1. Protein 1794 is 98% identical to A. thaliana UBQ10 (NP_974516) and 97% identical to a H. sapiens protein annotated as “similar to Ubiquitin-63E” (XP_001132949) and a P. troglodytes hypothetical protein (XP_001130911).

Additional components of the C. reinhardtii SUMOylation system are likely to emerge as studies of SUMOylation in other eukaryotes reveal new components. However, SUMOylation systems are often sufficiently unique that components in one organism may be missing in others. For example, the newly discovered RSUME protein found to enhance conjugation of SUMO-1, -2 and -3 to target proteins by binding to Ubc9 in mammalian cells (Carbia-Nagashima et al. 2007) has not been identified in our searches of the C. reinhardtii genome.

Stress—heat-shock, osmotic, nutrition, and photoautotrophic growth: Some SUMO target proteins are conjugated under normal growth conditions, while some are conjugated preferentially upon stresses (Johnson 2004; Miura et al. 2007). The conjugation
of SUMO to its target proteins may serve as a signal to guide regulation of expression of specific sets of genes under stress conditions (Kerscher 2007; Miura et al. 2007).

The accumulation of SUMO-conjugated proteins was detected when C. reinhardtii cells were shifted to 37° or 42° heat-shock conditions and to sorbitol- or NaCl-induced osmotic stresses (Figure 10, A and B, and supplemental Figure 6). The appearance of distinctly different sizes of SUMO-conjugated proteins produced under different stress conditions indicates that SUMOylation is involved in multiple cellular processes initiated by different environmental cues. Under each stress condition, 30 min of treatment induced more SUMO-conjugated proteins than 1 hr. This indicates that SUMOylation could be a rapid and transient reaction and that deSUMOylation may take place as soon as the signaling process is complete. Such a dynamic modification requires highly regulated SUMOylation and deSUMOylation enzymes.

Conclusions and future research: We have demonstrated the presence of SUMO and SUMO-conjugating systems in C. reinhardtii, a unicellular photosynthetic alga and model plant cell system. Free and conjugated SUMOs were detected by immunoblot analysis and shown to be localized in the nucleus by immunofluorescence. Endogenous free CrSUMO96 was purified by immunoprecipitation and identified by LC/MS/MS analysis. SCE (E2) of C. reinhardtii was cloned and shown to be functional in an in vivo E. coli SUMOylation system. Accumulation of SUMO-conjugated proteins was detected when the cells were subjected to environmental stresses, such as heat-shock and osmotic stresses.

Investigations in regard to how SUMO affects biological processes are only in their early stages (Johnson 2004; Kerscher et al. 2006; Geiss-Friedlander and Melchior 2007; Miura et al. 2007; Palanca\nc and Doye 2008). Identification of SUMO target proteins and an understanding of their biological functions in the SUMOylated and nonSUMOylated states lie ahead. The way forward will include challenges because (i) many SUMOylated proteins are present at a level below the normal detection limit (La et al. 2004), (ii) for most SUMO target proteins, only a small fraction of the substrate is SUMOylated at any given time, and (iii) there are strong SUMO protease (isopeptidase) activities in native cell lysates.

C. reinhardtii is a valuable model system to study various cellular functions and biochemical pathways because of its small genome size, haploid nature, susceptibility to gene manipulations, and, most importantly, the ability to grow the organism in large quantities (Weeks 1992; Rochaix et al. 1998). The present bioinformatics studies of the completed C. reinhardtii genome sequence have revealed much about SUMO-associated protein families in this organism and suggest that C. reinhardtii may be an especially facile organism for defining the role of SUMOylation in controlling gene expression and cellular functions such as response to environmental changes, photosynthesis, and flagellar-based motility.

The authors thank Richard Vierstra (University of Wisconsin) for the generous gift of antibodies to A. thaliana SUMO-1, Tom Elthon (University of Nebraska Protein Core Facility) for assistance with 2-D PAGE analyses, Susan Dutcher for providing procedures for microscopic detection of proteins using immunofluorescence, and Ashraf Raza (University of Nebraska Redox Biology Center) for assistance in analyzing tryptic fragments of CrSUMO96 by mass spectrometry. Systems administration by J. J. M. Riethe\non and F. Ma is gratefully acknowledged. This research was supported with funds from the U.S. National Science Foundation (IBN-0120802 to D.P.W.), the University of Nebraska Agricultural Research Division, and the National Science Foundation of China (no. 30500024 to Y.W.).

LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang et al., 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402.

Ballesteros, M. L., C. Bolle, L. M. Lois, J. M. Moore, J. P. Vielle-Calzada et al., 2001 LAF1, a MYB transcription activator for phytocrome A signaling. Genes Dev. 15: 2613–2625.

Bayer, P., A. Arndt, S. Metzger, R. Mahajan, F. Melchior et al., 1998 Structure determination of the ubiquitin-related modifier SUMO-1. J. Mol. Biol. 280: 275–286.

Bernier-Villamor, V., D. A. Sampson, M. J. Matunis and C. D. Lima, 2002 Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. Cell 108: 345–356.

Bieny, E. M. Clamp and R. Durbin, 2004 Genevis and genome-wise. Genome Res. 14: 988–995.

Broday, L., I. Koloteu, C. Didier, A. Bhoumik, B. G. Gupta et al., 2004 The small ubiquitin-like modifier (SUMO) is required for gonadal and uterine-ovulph morphogenesis in Caenorhabditis elegans. Genes Dev. 18: 2380–2391.

Cabrall, F. J., O. S. Pereira, Jr., C. S. Silva, R. Guerra-Sa and R. Vanderlee, 2008 Schistosoma mansoni encodes SMT3B and SMTC molecules responsible for post-translational modification of cellular proteins. Parasitol. Int. 57: 172–178.

Carbia-Nagashima, A., J. Gerez, C. Perez-Gastro, M. Paez-Perea, S. Silberstein et al., 2007 RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1α during hypoxia. Cell 131: 399–329.

Clamp, M., J. Cuff, S. M. Stearle and G. J. Barton, 2004 The Jalview Java alignment editor. Bioinformatics 20: 426–427.

Colby, T., A. Matthis, A. Boeckelmann and H.-P. Stubbe, 2006 SUMO-conjugating and SUMO-deconjugating enzymes from Arabidopsis. Plant Physiol. 142: 318–332.

Denison, C., A. D. Rudner, S. A. Gerber, C. E. Bakalarski, D. Moazed et al., 2004 A proteomic strategy for gaining insights into protein sumoylation in yeast. Mol. Cell. Proteomics 4: 246–254.

Desterro, J. M. P., J. Thomson and R. T. Hay, 1997 Ubc9 conjugates SUMO but not ubiquitin. FEBS Lett. 417: 297–300.

Eddy, S. R., 1998 Profile hidden Markov models. Bioinformatics 14: 755–763.

Ermolova, N. V., M. A. Cushman, T. Taybi, S. A. Condon, J. C. Cushman et al., 2003 Expression, purification, and initial characterization of a recombinant form of plant PEP-carboxylase kinase from CAM-induced Mesembryanthemum crystallinum with enhanced solubility in Escherichia coli. Protein Expr. Purif. 29: 125–131.

Finn, R. D., J. Tate, J. Mistry, P. C. Coggill, S. J. Sammut et al., 2008 The Pfam protein families database. Nucleic Acids Res. 36: D281–D288.

Gasco\nel, O., 1997 BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol. Biol. Evol. 14: 685–695.

Geiss-Friedlander, R., and F. Melchior, 2007 Concepts in sumoylation: a decade on. Nat. Rev. Mol. Cell Biol. 8: 947–956.


family of SUMO1/Smt3-specific isopeptidases and nuclear pores. Trends Biochem. Sci. 28: 612–618.

Miura, K., J. B. Jin and P. M. Hasegawa, 2007 Sumoylation, a post-translational regulatory process in plants. Curr. Opin. Plant Biol. 10: 495–502.

Murtaz, G., P. H. Reeves, Y-F. Fu, J. Bancroft, C. Dean et al., 2003 A nuclear protease required for flowering-time regulation in Arabidopsis reduces the abundance of small-ubiquitin-related-modifier conjugates. Plant Cell 15: 2308–2319.

Nishida, T., F. Kaneko, M. Kitagawa and H. Yasuda, 2001 Characterization of a novel mammalian SUMO-1/Smt3-specific isopeptidase, a homologue of rat axam, which is an axin-binding protein promoting β-catenin degradation. J. Biol. Chem. 276: 39060–39066.

Notredame, C., D. G. Higgins and J. Heringa, 2000 T-Coffee: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302: 205–217.

Palanca, B., and V. Doye, 2008 Sumoylating and desumoylating enzymes at nuclear pores: Underpinning their expected duties? Trends Cell Biol. 18: 174–183.

Pan, Z. Q., A. Kentsis, D. C. Dias, K. Yamah and K. Wu, 2004 Ned8 on cullin: building an expressway to protein degradation. Oncogene 15: 1985–1997.

Pickart, C. M., 2000 Ubiquitin in chains. Trends Biochem. Sci. 25: 54–58.

Pickart, C. M., 2001 Mechanisms underlying ubiquitination. Annu. Rev. Biochem. 70: 503–533.

Pickart, C. M., 2004 Back to the future with ubiquitin. Cell 116: 181–190.

Reeves, P. H., G. Murtaz, S. Dash and G. Coupland, 2002 Early in short days 4, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor FLC. Development 129: 5349–5361.

Rochaix, J.-D., M. Goldschmidt-Clermont and S. Merchant, 1998 The Molecular Biology of Chloroplasts and Mitochondria of Chlamydomonas. Kluwer Academic/Plenum Publishers, New York.

Rosas-Acosta, G., W. K. Russell, A. Seydoux, D. H. Russell and V. G. Wilson, 2005 A universal strategy for proteome studies of SUMO and other ubiquitin-like modifiers. Mol. Cell. Proteomics 4: 56–72.

Sampson, D. A., M. Wang and M. J. Murtaz, 2001 The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. J. Biol. Chem. 276: 21664–21669.

Schultz, J., F. Milpetz, P. Bork and C. P. Ponting, 1998 SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA 95: 5857–5864.

Schwarz, S. F. E., K. Matuschewski, D. Liaropoulos and M. Scheffner, 1999 The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme. Proc. Natl. Acad. Sci. USA 96: 559–564.

Seeler, J. S., and A. Dejean, 2003 Nuclear and unclear functions of SUMO. Nat. Rev. Mol. Cell Biol. 4: 690–699.

Spikes, D. A., J. Kramer, P. H. Bingham and K. Van Doren, 2003 The SWAP pre-mRNA splicing regulators are a novel, ancient protein family sharing a highly conserved sequence motif with the prp21 family of constitutive splicing factors. Nucleic Acids Res. 32: 4510–4519.

Stanke, M., and S. Waack, 2003 Gene prediction with a hidden Markov model and a new intron submodel. Bioinformatics 19(Suppl. 2): ii215–ii225.
Sternsdorf, T., K. Jensen, B. Reich and H. Will, 1999 The nuclear dot protein Sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. J. Biol. Chem. 274: 12555–12566.

Tanaka, K., J. Nishide, K. Okazaki, H. Kato, H. Niwa et al., 1999 Characterization of a fission yeast SUMO-1 homologue, Pmtp3, required for multiple nuclear events, including the control of telomere length and chromosome segregation. Mol. Cell. Biol. 19: 8660–8672.

Toshiaki, S., A. Ichyama, H. Saitoh, T. Kawakami, M. Omata et al., 1999 A new 30-kDa Ubiquitin-related SUMO-1 hydrolase from bovine brain. J. Biol. Chem. 44: 31131–31134.

Uchimura, Y., M. Nakao and H. Saitoh, 2004a Generation of SUMO-1 modified proteins in E. coli towards understanding the biochemistry/structural biology of the SUMO-1 pathway. FEBS Lett. 564: 85–90.

Uchimura, Y., M. Nakamura, K. Sugasawa, M. Nakao and H. Saitoh, 2004b Overproduction of eukaryotic SUMO-1 and SUMO-2-conjugated proteins in Escherichia coli. Anal. Biochem. 331: 204–206.

Weeks, D. P., 1992 Chlamydomonas: an increasingly powerful model plant cell system. Plant Cell 4: 871–878.

Weissman, A. M., 2001 Themes and variations on ubiquitylation. Nat. Rev. Mol. Cell Biol. 2: 169–178.

Wu, T. D., and C. K. Watanabe, 2005 GMAP: a genomic mapping and alignment program for mRNA and EST sequences. Bioinformatics 21: 1859–1875.

Xiang, Y., J. Zhang and D. P. Weeks, 2001 The Cia5 gene controls formation of the carbon concentrating mechanism in C. reinhardtii. Proc. Natl. Acad. Sci. USA 98: 5341–5346.

Yeh, E. T. H., L. Gong and T. Kamitani, 2000 Ubiquitin-like proteins: new wines in new bottles. Gene 248: 1–14.

Communicating editor: S. Dutcher