Identification and Characterization of a SUMO-1 Conjugation System That Modifies Neuronal Calcium/Calmodulin-dependent Protein Kinase II in \textit{Drosophila melanogaster}*

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\textit{Drosophila} Uba2 and Ubc9; SUMO-1 conjugation enzyme homologs (DmUba2 and DmUbc9) were isolated as calcium/calmodulin-dependent kinase II (CaMKII) interacting proteins by yeast two-hybrid screening of an adult head cDNA library. We found that at least one isofrom of \textit{Drosophila} neuronal CaMKII is conjugated to DmSUMO-1 \textit{in vivo}. The interactions observed in the two-hybrid screen may therefore reflect catalytic events. To understand the role of SUMO conjugation in the brain, we undertook a characterization of the system. The other required components of the system, \textit{Drosophila} Aos1 and SUMO-1 (DmAos1 and DmSUMO-1), were identified in expressed sequence tag data base searches. Purified recombinant DmUba2/DmAos1 dimer can activate DmSUMO-1 \textit{in vitro} and transfer DmSUMO-1 to recombinant DmUbc9. DmSUMO-1 conjugation occurs in all developmental stages of \textit{Drosophila} and in the adult central nervous system. Overexpression of a putative dominant negative DmUba2(C175S) mutant protein in the \textit{Drosophila} central nervous system resulted in an increase in overall DmSUMO-1 conjugates and a base-sensitive p120 species, which is likely to be DmUba2(C175S) linked to endogenous DmSUMO-1 through an oxygen ester bond. Overexpression of DmUba2(wt) protein \textit{in vivo} also led to increased levels of DmSUMO-1 conjugates. High level overexpression of either DmUba2(wt) or DmUba2(C175S) in the \textit{Drosophila} central nervous system caused pupal and earlier stage lethality. Expression in the developing eye led to a rough eye phenotype with retinal degeneration. These results suggest that normal SUMO conjugation is essential in the differentiated nervous system and reveal a potential novel mechanism that regulates neuronal calcium/calmodulin-dependent protein kinase II function.

Covalent post-translational modification of proteins by small polypeptides is an important regulatory mechanism. The best studied molecule of this type is ubiquitin. Ubiquitination primed, proteosome-mediated protein degradation is involved in multiple cellular processes (1–3). In the initial ubiquitin conjugation step, an activating enzyme (ubiquitin activating enzyme 1) utilizes ATP to activate ubiquitin C terminus and binds to ubiquitin with an active site cysteine residue through a thioester linkage. Activated ubiquitin is then transferred to one of the ubiquitin conjugating enzymes (Ubc) through threonylation. Ubcs can directly (or in some cases, with the help of ubiquitin protein ligases) catalyze the isopeptidyl bond formation between ubiquitin C terminus and lysine residues in protein substrates. Polyubiquitinated proteins are recognized and degraded by a multisubunit 26 S proteosome complex.

A family of small modifiers bearing sequence homology to ubiquitin, the SUMO (small ubiquitin-related modifier) family, has been identified recently (4–13) and gained much attention. The \textit{S. cerevisiae} homolog in the family, Smt3, was originally identified as a suppressor of a temperature-sensitive mutation of \textit{MIF2}, which encodes a centromere-binding protein (13). The human homolog, SUMO-1, was identified as a ubiquitin-related modifier conjugated to RanGAP1 (7, 8) and an interaction partner of Fas (6), Rad51/Rad52 (14), and PML (4). While having less than 20% identity in primary sequence to ubiquitin, SUMO’s conjugation mechanism parallels that of ubiquitin in remarkable ways. Two \textit{Saccharomyces cerevisiae} SUMO proteins, Uba2 (15) and Aos1 (16), were shown to form a heterodimer (structurally equivalent to a ubiquitin activating enzyme 1) and function to activate the mature form of Smt3 (16). Ubc9, a gene product required for \textit{S. cerevisiae} \textit{G}_{2}-M transition (17), was shown to function as an Smt3 conjugating enzyme (18). An Smt3-specific protease, Ulpl, has been recently cloned and shown to contribute to the maturation of Smt3 precursor by cleaving after and exposing the mature end glycine residue and to cleave Smt3 from Smt3-protein conjugates (19). Mammalian Ubc9 was also shown to be a SUMO-1 specific conjugating enzyme (20–23). The activating enzyme complex was recently isolated for human (24). It was shown that recombinant human Uba2, Aos1, and Ubc9 are sufficient to conjugate SUMO-1 to a human substrate, IκBα (24).

SUMO modification has several unique features. Subcellular fractionation experiments in mammalian cells showed that proteins that can form conjugates with SUMO are limited and are mostly high molecular weight nuclear proteins (7, 8, 25). Confirmed substrates include RanGAP1 (7, 8, 20, 22), PML (9, 26, 27), Cdc3 (28), and IκBα (29). Since none of the conserved lysine residues imperative for ubiquitin polymer formation are present in SUMO, SUMO is probably not able to form polymeric chains. Identified functions associated with SUMO modification include: causing changes in substate subcellular localization (RanGAP1, dorsal, and PML) (7, 8, 27, 30),

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\footnotesize{† The abbreviations used are: Ubc, ubiquitin conjugation enzyme; CaMKII, calcium/calmodulin-dependent protein kinase II; EST, expressed sequence tag; BME, 2-mercaptoethanol; NEM, N-ethylmaleimide; SUMO, small ubiquitin-related modifier; bp, base pair(s); PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.}
promoting septin ring formation (Cdc3) (28), and inhibiting a substrate’s ubiquitination and subsequent degradation (LeBo) (29).

We report the identification of the Drosophila Uba2, Aos1, and SUMO-1 homologs through a combination of yeast two-hybrid screening and Drosophila expressed sequence tag (EST) database searches. Both the sequences of these components and the mechanism through which SUMO-1 is activated are found to be highly conserved in Drosophila. We present in vivo evidence supporting Drosophila Uba2 functioning to activate the Drosophila SUMO-1, and for this conjugation system playing a critical role in the nervous system. In addition, we found that at least one isoform of Drosophila neuronal CaMKII is modified by DmSUMO-1.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The yeast two-hybrid screen was performed according to a protocol described previously (31). In brief, a Drosophila calciu/calmodulin-dependent kinase II (CaMKII) R5 isoform (32) variable region plus association domain cDNA was inserted into a pJG4-5 vector and used as a bait to screen a Drosophila expressed cDNA library (provided by J. Huang and M. Rosbash, Brandeis University, Waltham, MA) in vector pJG4-5. The bait and the library plasmids were sequentially transformed into the host strain EGY48 (containing an integrated LEU2 reporter gene with its normal upstream regulatory sequences replaced with LexA operators) and plated onto solid medium to select for the lacZ reporter plasmid, the bait, and the library plasmids. Transformants were collected and an aliquot (corresponding to 25 million transformants) was replated on solid medium to select for induced transcription of the LEU2 reporter. Among the 1500 Leu+ transformants that appeared, 100 were eliminated due to their ability to activate the lacZ reporter on selective medium containing glucose. The rest of the transformants were visually classified into strong, medium, and weak groups according to their speed to turn blue on galactose-5-chloro-3-iodo-2'-deoxyuridine (CId) medium. 200 transformants that belonged to the strong group were further analyzed: library plasmids were retrieved from these transformants and the cDNA inserts classified according to their HasIII digestion patterns. Representative clones from each class were sequenced using the ABI prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems) and the Biochemistry Core Facility at Brandeis University.

Isolation and Identification of Full-length cDNA Clones and Amplification of the Coding Regions—The EcoRI fragment of interaction clone XL2 insert contains DmUbC9's full-length coding region and part of its 3’-untranslated region. This fragment was used for the fusion construct and probe labeling described below.

To obtain DmUbC2 cDNA, the 5’-nucleotide sequence of interaction clone XL48 was used to search the Berkeley Drosophila Genome Project (BDGP) EST database. One of the LD clones thus identified (LD11358) was found to contain the full-length DmUbA2 coding region and flanking regions. To introduce the C175S mutation, identified (LD11358) was found to contain the full-length DmUba2 and DmAos1 full-length cDNA encoding plasmids (in the experiment to test complex formation between Hi6-DmUba2 and GST-DmAos1, the bacteria harbor both a GST fusion protein encoding a His-tag protein encoding plasmid) was diluted to an A260 of ~0.1 in LB medium containing 50 μg/ml carbenicillin, grown at 37 °C to an A660 of ~0.4. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.1–0.5 mM and the culture was allowed to grow for 2.5 h under the induction condition. Cells were harvested by centrifugation at 4 °C. Pellets were lysed on ice by sonication in lysis buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.1% Triton, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). Lysate was centrifuged and the supernatant was mixed with glutathione-Sepharose (Amersham Pharma Biotech) and allowed to bind at 4 °C for 30 min. Glutathione-Sepharose was pelleted by low speed centrifugation and then washed extensively in wash buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 0.1% Triton). The bound GST fusion protein was eluted with 10–20 mM reduced glutathione (Amersham Pharma Biotech) in 100 mM Tris-HCl, pH 8.0. In some cases, glutathione was subsequently removed through dialysis.

GST-K-DmSUMO-1 was radioactively labeled according to a previous protocol (35) with some modification. Briefly, the GST fusion protein was bound to glutathione-Sepharose beads and washed once with labeling buffer (50 mM sodium phosphate, pH 7.15, 10 mM MgCl2, 5 mM NaF, and 1 mM dithiothreitol). The supernatant was aspirated and the Sepharose beads were resuspended in 2–3 bed volumes of the labeling buffer containing 0.5 unit/μl of the catalytic subunit of protein kinase A (Sigma) and 0.7 μCi/μl 125I-labeled ATP (3000 Ci/mmol, 10 μCi/ml, PerkinElmer Life Sciences). The kinase reaction proceeded at 30 °C for 30 min. The reaction was terminated by incubation in gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 0.1 mg/ml of each protease inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). The resulting kinase reaction mixture was applied to SDS-PAGE. The gel was stained with Coomassie Blue. For Ni-NTA-agarose purification, the His6-DmSUMO-1 complex was eluted in 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. This eluate was also used for in vitro SUMO-1 activation assay. To assess complex formation, eluate was treated at 37 °C for 10 min with SDS sample buffer containing or lacking BME before loading onto an SDS-PAGE. The gel was stained with Coomassie Blue. For Ni-NTA-agarose pull down, bacterial pellets were lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Triton, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM MgCl2, and 5 mM BME through sonication. Clarified lysates were supplemented with 20 mM imidazole. Ni-NTA-agarose was added and the binding was allowed to proceed for 25 min. Extensive washing was done with a buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Triton, 1 mM MgCl2, 5 mM BME, and 20 mM imidazole. Elution was done in a buffer containing 200 mM imidazole in 10 mM Tris-HCl, pH 7.0, 100 mM NaCl, and 1 mM MgCl2. To assess complex formation, eluate was treated at 37 °C for 10 min with SDS sample buffer containing or lacking BME before loading for SDS-PAGE. The gel was stained with Coomassie Blue.

Generation of Antisera—Antisera were produced using GST fusion proteins overexpressed in and purified from E. coli as antigens. The following fusion proteins were used: GST-XL7 (XL7 encodes the C-terminal 286 amino acids of DmUbA2), GST-DmAos1, GST-DmAos9, GST-K-DmSUMO-1, and GST-CaMKII-C (CaMKII-C encodes the N-terminal 265 amino acids of Drosophila CaMKII). Antisera thus ob-
tained were used directly for immunoblotting analysis.

Fly Extract Preparation—In experiments estimating protein expression levels during fly development and comparing expression levels in adult heads versus bodies, appropriate amounts of different stage flies were collected (adult fly heads and bodies were separated with a sizing sieve and a rotor to remove flies). Fresh fly heads were disrupted in grinding buffer (40 mM Tris-HCl, pH 7.5, 0.1 mM MgCl₂, 1% Triton-100, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) with a tissue grinder (Concept Inc.). Ground samples were centrifuged and the supernatant was used in standard immunoblotting. The pellet was resuspended in a buffer containing 25 mM Tris, pH 7.5, 150 mM NaCl, and 10 or 50 mM N-ethylmaleimide (NEM). NEM was used in the purification procedure to inhibit SUMO-specific isopeptidases (7). For the control sample, the buffer did not contain NEM. Homogenates were spun at 4 °C to remove cuticle. Saturated ammonium sulfate was slowly added into the supernatant with stirring to a final concentration of 45%. Staining continued for 30 min on ice, after which samples were precipitated by adding 10 ml for 8000 rpm. The pellet was resuspended in 3 ml of buffer containing 25 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, 0.5 mM CaCl₂, and 0, 10, or 50 mM NEM. CaM-Sepharose pre-equilibrated with a buffer containing 25 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, and 0.5 mM CaCl₂ was added. Binding was allowed to proceed for 1 h. CaM-Sepharose was then pelleted at room temperature through low-speed centrifugation, and a small fraction of the supernatant was used to be the total sample in immunoblotting. The pellet Sepharose was extensively washed in a buffer containing 25 mM Tris, pH 7.5, 10% glycerol, 500 mM NaCl, and 0.5 mM CaCl₂, Bound proteins were eluted at 100 °C in SDS sample buffer. Boiled heads were spun and the supernatant used in standard immunoblotting.

Detection of DmSUMO-1 Conjugates in Mammalian Cells—In the experiment to assess DmSUMO-1 conjugation to mammalian proteins, COS cells transfected with pcDNA3-HA (control), pcDNA3-HA-DmSUMO-1 (encoding the putative mature form of DmSUMO-1), or pcDNA3-HA-DmSUMO1FL (encoding the full-length DmSUMO-1) were pelleted (~30 h post-transfection), resuspended in a buffer containing 10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 μg/ml aprotinin, and allowed to sit on ice for 15 min. The lysates were cleared by centrifugation and used as crude cytosolic fractions. The pellets after centrifugation were resuspended in a buffer containing 10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 5% Nonidet P-40, and further dissolved in SDS sample buffer. The fractions thus obtained were used as crude nuclear fractions. Proteins were separated by SDS-PAGE in standard immunoblotting using an anti-HA monoclonal antibody (provided by L. Liu and M. Rosbash, Brandeis University, Waltham, MA).

DmSUMO-1 Thioester Formation Assays—In experiments using fresh fly head extract as a source for DmSUMO-1 activating enzymes, extracts were prepared by grinding Canton-S fly heads in a buffer containing 50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride with an electronic tissue grinder. Cuticle was removed through centrifugation and the supernatant was immediately used in in vitro thioester formation assays. In experiments using purified recombinant proteins as the activating enzyme source, the eluate (containing His-DmUba2/GST-DmAos1 heterodimer) from glutathione-Sepharose beads was used. A typical thioester formation assay was done in a 20-μl volume and contained ~2.5 μM radioactively labeled GST-K-DmSUMO-1, 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM MgCl₂, 0.1 mM dithiothreitol and some of the following: ~100 mM equivalent of recombinant His6-DmUba2GST-DmAos1 complex (or fresh head extract containing 50 μg of total protein), 0.1 μM recombinant GST-DmUbc9 and 5 mM ATP. Reactions were allowed to proceed at room temperature for 30–45 min. Each reaction mixture was then split into two tubes, incubated at 37 °C for 10–15 min in SDS sample buffer containing or lacking BME, and followed by SDS-PAGE and autoradiography.

Germ line Transformation, Fly Stocks, Genetic Crosses, and Phenotypic Scoring—A element mediated germ line transformation was performed by standard methods by injecting DNA into a Δ2–3 line (38). pUAST-DmUba2(Δ2) and pUAST-DmUba2(Δ175S) were used to generate fly lines bearing UAS-DmUba2(Δ2) and UAS-DmUba2(Δ175S) transgenes in their genome, respectively. Insertions were mapped and transposase removed genetically. After mapping, single insertion lines were outcrossed to Dr1/1w for at least seven generations to exchange the genetic background to that of Dr1/1w. All stocks were maintained in a homozygous state, indicating that none of the insertions were lethal due to positional effects.

The pan-neural GAL4 driver line C155 (39) was used to drive transgene expression in fly neurons. This driver (on X-chromosome) shows both dosage compensation (GAL4 is expressed more in males than females) and temperature dependence (GAL4 is more stable at 29 °C than at 25 °C). This line drives strong expression in all neurons. Neuronal expression persists from embryo through all later stages. To examine lethality caused by overexpressing the transgenes in neurons, homozygous autosomal single insert transgenic line males were crossed to the C155 females at 29 °C. The number of male progeny was divided by the number of female progeny (in which expression was not strong enough to be lethal) for each cross to yield an estimated lethality number for each line. To overexpress two copies of transgenes, a homozgyous second chromosomal insertion line was crossed to a homozgyous third chromosomal insertion line at 25 °C. Progeny from this cross were crossed to strain w; CyO; +/+ Male progeny from this cross, progeny from the second cross, and single insertions (identified by absence of a white eye color) were crossed to C155 females at 25 or 29 °C. Progeny from this last cross were classified according to wing phenotype (wt or Xa) and scored.

An autosomal GMR-GAL4 line was used to drive expression in the fly eye at 29 °C. This GAL4 line drives high level expression in all cells of the differentiated eye (40). Progeny of control crosses to Dr1/1w were crossed with progeny of GMR-GAL4 × transgene crosses by light microscopy.

Fly Head Extract Preparation and Base Treatment—To detect DmUba2(Δ2) or DmUba2(Δ175S) expression from transgenes and compare DmSUMO-1 conjugate levels in C155/Dr1/1w control flies and C155/Dr1/1w; UAS-DmUba2/+ flies, progeny from appropriate crosses were collected immediately after eclosion. Fresh, manually severed heads were ground in grinding buffer, supplemented with SDS sample buffer, and heated at 100 °C for 10 min. The immediate addition of SDS sample buffer and heating can presumably inactivate proteases, including the proteases that are specific for DmSUMO-1-substrate isopeptide bond. Cuticle was removed through centrifugation. Total protein amount in the supernatant was quantitated using the Bio-Rad protein assay reagent (Bio-Rad). Extracts were fractionated by SDS-PAGE and treated as in standard immunoblotting. In base treatment experiments, the above cleared extracts were briefly (30 s to 5 min) incubated in 150 mM NaOH at room temperature, fractionated by SDS-PAGE, and treated as in standard immunoblotting.

RESULTS

Isolation of the Drosophila Uba9 and Uba2 Homologs—In a yeast two-hybrid screen to isolate proteins that interact with CaMKII, we isolated three clones from a Drosophila adult head cDNA library that encode polypeptides whose sequences suggested that they functioned as ubiquitin or related conjugation system components. Interaction clone XL2 encodes a recently
reported *Drosophila* Ubc9 homolog (41, 42). XL2 contained a complete open reading frame encoding a protein with high homology to Ubc9s identified in human (83% identical) (43) and yeast (55% identical) (17). The 477-bp *Drosophila* Ubc9 (DmUbc9) open reading frame encodes a 159-amino acid protein with a calculated molecular mass of 18 kDa. The Ubc consensus motif (F/Y/W)HPN(I/V/L)(D/S)(I/V/L)(I/L) (44) containing the active site cysteine (underlined) is conserved in DmUbc9.

Interaction clone XL48 contained a cDNA sequence with a 504-amino acid open reading frame. Interaction clone XL7 encoded the C-terminal 266 amino acids of XL48. An NCBI Blast search showed extensive homology between the XL48-encoded peptide and the C terminus of yeast Uba2 (ScUba2). In situ hybridization using biotin-labeled XL7 cDNA probes on *Drosophila* third instar larval salivary gland polytene chromosomes localized the DmUba2 gene to arm 3L region 65B5-C3 (data not shown).

A *Drosophila* EST data base search yielded multiple LD (adult head library) and other library cDNA clones that were exact matches to the 5′ cDNA sequence of clone XL48. A representative clone, LD11358, was obtained from the clone distributor and found to contain a 2100-bp open reading frame encoding a 700-amino acid protein. The protein has a calculated molecular mass of 78 kDa. Sequence alignment (Fig. 1A) shows that the N-terminal 200-amino acid stretch is highly homologous (48% identical) to the corresponding region (domains I-III) in ScUba2 (16). The active site cysteine (Cys175) and its immediate neighboring sequence are conserved, as is the putative nucleotide-binding site (GXGXXG). The C-terminal 150-amino acid region contains a potential nuclear localization signal sequence: KRKR. Sequence similarity, peptide length, and domain arrangement all suggest strongly that we have identified a *Drosophila* Uba2 homolog. The protein encoded by LD11358 was thus designated *Drosophila* Uba2 (DmUba2).

**Identification of the Drosophila Aos1 Homolog (DmAos1)—** The existence of DmAos2 and DmUbc9 suggested that there should also be Aos1 and SUMO-1 homologs in the *Drosophila* genome. To search for a *Drosophila* Aos1 homolog, we used the yeast Aos1 (ScAos1) protein sequence to BLAST the BDGP data base. The N terminus of the polypeptide encoded by one class of EST clones (represented by LD13875) was found to have significant homology (34% identity) with the N-terminal 160 amino acids (Domain I) of ScAos1 (16). Upon sequencing LD13875, we found that it encodes a 337-amino acid protein, with a calculated molecular mass of 38 kDa. Sequence alignment (Fig. 1B) indicates that the C-terminal region (45 amino acids) of the protein shares 55% identity with a corresponding region in ScAos1, which is part of the designated Domain II (16). We therefore named the protein encoded by LD13875 *Drosophila* Aos1 (DmAos1). A BDGP data base search using DmAos1 cDNA sequence localized the DmAos1 gene to chromosome 3R at 87B.

**Identification of the Drosophila SUMO-1 Homolog (DmSUMO-1)—** To identify the *Drosophila* SUMO-1 homolog, we used the yeast Smt3 protein sequence (GenBank™ U727233) to search the BDGP EST data base for high homology matches. A single EST clone (LD07775) was identified which encodes a 90-amino acid protein with 41% identity to Smt3. The protein thus identified was named *Drosophila* SUMO-1 (DmSUMO-1). Since no in-frame stop codon was found 5′ to the first ATG in the LD07775 sequence, Northern blotting was done with fly head total RNA using LD07775 cDNA as a probe to confirm that the cDNA has a similar size to the DmSUMO-1 mRNA (data not shown). Sequence alignment (Fig. 1C) revealed higher homology between DmSUMO-1 and human SUMO-1: hSmt3A (56% identical), hSmt3B (56% identical), and SUMO-1/hSmt3C (50% identical). The double glycine, which is critically involved in maturation and conjugation (16) of SUMO-1 family members, is also conserved in DmSUMO-1. A putative *Drosophila* Ulp1 homolog (19) which is thought to cleave after Gly35 and convert DmSUMO-1 to its mature form, has also been identified.

**CaMKII Does Not Form Stable Complexes with DmUba2 or DmUbc9—** Isolation of DmUba2 and DmUbc9 as potential CaMKII-interacting proteins was interesting but needed independent biochemical confirmation. Two main approaches were taken to identify stable complex formation: coimmunoprecipitation assays and GST fusion protein pull down assays. In the immunoprecipitation assay, we co-transfected cDNAs encoding the two proteins tested into a mammalian cell line. This was followed by immunoprecipitation (using an antibody against one of the proteins or its epitope tag) and immunoblotting (using an antibody against the other protein or its epitope tag) to determine if stable complexes had formed. In the GST fusion protein pull down assay, we produced one of the tested proteins as a GST fusion protein in *E. coli*, purified it, and immobilized it on glutathione-Sepharose. Mammalian cell lysate containing the overproduced second protein was then incubated with the solid matrix and retention of the second protein on the matrix was tested afterward with immunoblotting. Both approaches yielded negative results (data not shown).

**CaMKII Is a Substrate for SUMO Conjugation—** We were interested in the possibility that the interactions between CaMKII and DmUba2 or DmUbc9 observed in the original yeast two-hybrid screen assay might be involved in catalytic functions and are transient. Such interactions could involve regions close to the enzyme catalytic site and regions close to the modification site on the substrate, or they could involve regions that are comparatively remote from where catalysis occurs. Either type of contact could contribute to the overall interaction between the enzyme and the substrate. More specifically, we were interested in the possibility that CaMKII could be modified by DmSUMO-1. To test this possibility we set out to determine if any of the CaMKII isoforms were SUMO conjugated in vivo.

A polyclonal antiserum raised against the catalytic domain of CaMKII could recognize, in fly head extract, multiple signals in the 50–60-kDa region (Fig. 2A, lanes 1–3). These bands correspond to the predicted sizes of cloned isoforms of CaMKII (32). The antiserum also recognized a minor signal of 70 kDa (p70) which is larger than any cloned or predicted CaMKII splice forms (Fig. 2A, lanes 1–3). In addition, the antiserum recognized a 45-kDa signal (p45) in fly head extract (Fig. 2A, lanes 1–3), which could be a degradation product of CaMKII.

To determine if these immunoreactive bands were actually forms of CaMKII, the kinase was partially purified from fly heads by affinity chromatography on CaM-Sepharose. The purified CaMKII fraction contained not only the expected 50–60-kDa isoforms, but also contained immunoreactive bands of both higher (70 kDa) and lower (39–45 kDa) molecular masses (Fig. 2, A, lanes 4–6; B, lanes 1 and 2). These signals, which were seen at a low level in the total head extract, were enriched by the CaM-Sepharose purification procedure, suggesting that they are indeed forms of CaMKII.

To determine if any of the CaMKII forms we had purified were SUMO conjugates, we performed an immunoblot on the purified material using anti-DmSUMO-1 antiserum. Head extracts were prepared using several concentrations of NEM,
FIG. 1. Identification of the Drosophila Uba2 (DmUba2), Aos1 (DmAos1), and SUMO-1 (DmSUMO-1) proteins. A, protein sequence comparisons of DmUba2 with S. cerevisiae Uba2 (ScUba2, GenBank™ U32274) and human Uba2 (HsUba2, GenBank™ AF110957). Sequences were aligned using the PileUp program (GCG package, Genetics Computer Group, Madison, WI). Residues identical among at least two of the proteins are shaded dark and appear as white characters. Residues showing conservative substitution among at least two of the proteins are shaded gray (if not already in dark). Overall, DmUba2 is 33% identical to ScUba2 and 49% identical to HsUba2. Higher homology occurs in the N-terminal ~200 amino acids (the putative domain I+II). In this region, DmUba2 is 48% identical to ScUba2 and 72% identical to HsUba2. B, protein sequence comparisons of DmAos1 with S. cerevisiae Aos1 (ScAos1, YPR180W), the putative Schizosaccharomyces pombe Aos1 (SpAos1, EMBL Y08805), and human Aos1 (HaAos1, GenBank™ AF110956). Sequences were aligned using the PileUp program. Residues identical among at least two of the proteins are shaded dark and appear as white characters. Residues showing conservative substitution among at least two of the proteins are shaded gray (if not already in dark). Overall, DmAos1 is 24% identical to ScAos1, 22% identical to SpAos1, and 34% identical to HaAos1. Higher homology occurs in the N-terminal ~170 amino acids (putative domain I). In this region, DmAos1 is 34% identical to ScAos1, 29% identical to SpAos1, and 40% identical to HaAos1. C, protein sequence comparisons of DmSUMO-1, human SUMO-1 (HsSUMO-1, GenBank™ U67122), human Smt3A (HsSmt3A, GenBank™ X98584), human Smt3B (HsSmt3B, GenBank™ L76416), S. cerevisiae Smt3 (ScSmt3, GenBank™ U27233), Caenorhabditis elegans Smt3 (CeSmt3, GenBank™ U94830), and Drosophila Ubiquitin (DmUb, GenBank™ M22428). Sequences were aligned using the PileUp program. Residues identical among at least four of the proteins are shaded dark and appear as white characters. Residues showing conservative substitution among at least four of the proteins are shaded gray (if not already in dark). Overall, DmSUMO-1 is 50% identical to HsSUMO-1, 56% identical to HsSmt3A, 56% identical to HsSmt3B, 41% identical to ScSmt3, 46% identical to CeSmt3, and 10% identical to DmUb.
SUMO-1 Modification of CaMKII in Drosophila

**Western Blot:**
- **anti-CaMKII**
- **anti-DmSUMO-1**

**FIG. 2. Neuronal CaMKII is modified by DmSUMO-1.** A, partial purification of Drosophila neuronal CaMKII. CaMKII was purified from fly heads using CaM-Sepharose as described under “Experimental Procedures.” Total fly head extract (lanes 1–3) and proteins bound to CaM-Sepharose (lanes 4–6) were separated by SDS-PAGE and immunoblotted with anti-CaMKII antibody made against the catalytic domain of the kinase. The concentration of NEM used in extract preparation and later purification steps is indicated below each lane. In addition to the major (triplet) CaMKII signals in the 50–60-kDa region, a 70-kDa signal (p70) and a 45-kDa (p45) signal could be recognized by the anti-CaMKII antibody in the total fly head extract (lanes 1–3). p45 is likely a degradation product of CaMKII. As discussed in the text and shown in B, p70 is the DmSUMO-1 conjugate of at least one CaMKII isoform. In the CaM-Sepharose eluates (lanes 4–6), p70 and p45, are enriched and signals at 39–40 kDa are also visible (lane 5). Overexposure of the immunoblotting membrane indicates that p39 and p40 also exist at a low level in total extract (not shown). As discussed in the text and shown in B, p40 is likely a degradation product of p70. The lack of this signal in lanes 1 and 4 indicates that NEM affects the degradation of p70 or p40 in the extract. B, CaMKII is modified by DmSUMO-1. Partially purified CaMKII was separated on SDS-PAGE and immunoblotted with anti-CaMKII antisera (lanes 1, 2), or anti-DmSUMO-1 antisera (lanes 3 and 4). The concentration of NEM used in extract preparation and later purification steps is labeled below each lane. p70 can be recognized by both antisera. The p58 signal in lane 4 is likely a degradation product of p70. p40 in lanes 2 and 4 is also likely a p70 degradation product which contains DmSUMO-1 linked to a region that spans at least part of the catalytic domain.

which at high concentrations is an inhibitor of SUMO-specific isopeptidases (7). We found that p70 was able to bind to CaM-Sepharose, was recognized by the anti-CaMKII antisera (Fig. 2A, lanes 4–6), and could also be recognized by the anti-DmSUMO-1 antisera (Fig. 2B, lanes 3 and 4). In addition, the amount of p70 present was enhanced by the presence of NEM, suggesting it is a SUMO conjugate. The molecular weight of p70 is consistent with one molecule of DmSUMO-1 conjugated to one subunit of CaMKII.

Another NEM-specific signal, p40, was also enriched by CaM-Sepharose purification. This signal exists at a low level in NEM-treated head extract but not in untreated extract (not shown). Since p40 can be recognized by both anti-CaMKII and anti-DmSUMO-1 antisera (Fig. 2B, lanes 2 and 4), it is likely to be a p70 degradation product which contains DmSUMO-1 linked to a region that spans at least part of the CaMKII catalytic domain. In agreement with this, p40 cannot be recognized by an antisera raised against the C terminus of CaMKII (data not shown).

These data show that at least one isoform of neuronal CaMKII is modified by DmSUMO-1 and suggest that the interactions observed between CaMKII and DmAos1 and DmUba2 and DmUba2 in the original two-hybrid assay are catalytic ones. Since some CaMKII isoforms have very similar molecular weights, it is formally possible that p70 is constituted of two or more modified isoforms. The function of this modification is currently unknown.

**SUMO Conjugation Components Are Enriched in Adult Nervous System—**The role of SUMO in the function of the differentiated nervous system has not previously been addressed. The identification of CaMKII, an important neuronal signal transduction molecule, as a substrate for this modification system suggests the possibility that SUMO conjugation may be important for adult function of the nervous system. To begin to investigate this we have characterized the Drosophila nervous system SUMO-conjugation pathway. Polyclonal antiserum raised against recombinant GST-XL7 (DmUba2) could recognize, in fly extracts, a major signal at 95 kDa (Fig. 3A) and several minor signals (see Fig. 7A, lane 1). The 95-kDa signal is apparently DmAos1; ScUba2 was previously also shown to run at a significantly higher position than its calculated molecular mass and the reason for this anomalous mobility remains unknown (15). Signals lower than 95 kDa could either be DmUba2...
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degradation products or peptides that cross-react with the antiserum. In experiments where relatively long exposure was applied in immunoblotting, we noticed that several species with molecular mass higher than 95 kDa could also be weakly recognized by this antiserum (Fig. 7B, lane 1). These could be modified forms of DmUba2 (see below). In immunoblots of crude extracts prepared from different developmental stages, we observed that DmUba2 is expressed in all stages examined (Fig. 3A). Embryos contain an estimated 5–10-fold higher level of this protein than tissues in other stages examined. The protein is expressed in both adult heads and bodies at a comparable level (Fig. 3B).

DmUbc9 was found to be significantly enriched in adult fly heads versus bodies, both at the transcriptional level (−3-fold difference) (Fig. 3C) and protein level (−10-fold difference) (Fig. 3B), suggesting a potentially important role of DmUbc9 in adult central nervous system function.

Antiserum raised against recombinant GST-DmAos1 could recognize a major signal at 40 kDa in Canton-S head extract (Fig. 3A). Like DmUba2, DmAos1 is expressed in all developmental stages examined (Fig. 3A). Embryos contain a much higher level of this protein than all the other developmental stages examined. Adult heads and bodies contain comparable levels of this protein (Fig. 3B).

Polyclonal antiserum raised against recombinant GST-K-DmSUMO-1 could recognize a major signal at the 19-kDa position (Figs. 3B and 7C, lane 1), which is apparently the DmSUMO-1 monomer. In addition, the antiserum could recognize some proteins at higher molecular weight range (see Fig. 7C, lane 1). The staining profile obtained looked similar to Smt3 conjugate patterns (16) and different from a profile resulting from cross-reaction of proteins with an unrelated antiserum, which usually gave a more spread-out and random collection of bands. This suggests that the signals recognized by our DmSUMO-1 antiserum consist of endogenous fly head proteins which are conjugated to DmSUMO-1. DmSUMO-1 expression and conjugation were observed for all developmental stage flies examined (data not shown). Slight conjugation pattern differences exist for different developmental stages (data not shown), reflecting availability or preferential modification of certain substrates. DmSUMO-1 is also expressed (Fig. 3B) and conjugated (data not shown) in adult bodies.

DmSUMO-1 and DmUbc9 Can Be Utilized by Mammalian and Yeast SUMO Conjugation Enzymes—To confirm that the proteins we had cloned were functional in SUMO conjugation, we utilized expression in heterologous systems. When expressed in COS cells, both HA-DmSUMO-1 (DmSUMO-1 ending at Gly188) and HA-DmSUMO-1FL (DmSUMO-1 full-length protein) were able to be conjugated to high molecular weight mammalian proteins, as an anti-HA monoclonal antibody could specifically recognize (in immunoblotting) several signals (>100 kDa) in cells transfected with constructs encoding HA-DmSUMO-1 and HA-DmSUMO-1FL but not in cells transfected with the vector (Fig. 4), suggesting that DmSUMO-1 can be recognized and utilized by mammalian SUMO-1 processing/conjugation machinery to function as a protein modifier.

DmUbc9 is also able to function in a heterologous SUMO conjugation system. Overexpression of DmUbc9 could rescue the growth defect of a Ubc9 temperature-sensitive mutation-bearing yeast strain (17) at restrictive temperature (data not shown and Ref. 42). These data support the identification of these proteins as genuine SUMO conjugation system components.

DmUba2 and DmAos1 Can Form a Complex—Function of Uba2 and Aos1 as a SUMO E1 is thought to require dimerization. We tested the ability of DmUba2 and DmAos1 to form a complex. When His6-DmUba2 and GST-DmAos1 were coexpressed in E. coli, we were able to use Ni-NTA-agarose to pull down a complex of His6-DmUba2-GST-DmAos1 with a stoichiometry close to 1:1 (Fig. 5A, lanes 3 and 4), which agrees with the gel filtration experiments showing that the yeast Uba2 and Aos1 form a heterodimer (16). We were also able to use glutathione-Sepharose to pull down His6-DmUba2 (Fig. 5A, lanes 1 and 2). We noticed that under both pull down conditions a significant amount of His6-DmUba2 ran at ~140-kDa position when no reducing agent was added into the eluates before gel loading (Fig. 5A, lanes 1 and 3, see “Experimental Procedures“). When the samples were treated with BME after elution, most of the p140 species could be converted into the p100 species corresponding to monomeric His6-DmUba2 (Fig. 5A, lanes 2 and 4). The p140 species may be caused by an intramolecular disulfide linkage which changes the conformation and mobility of the protein.

To determine the subcellular localization of DmUba2, we examined GFP-DmUba2 expressed in COS cells. GFP-DmUba2 localized exclusively within the nuclei (Fig. 5B, panel 1). GFP-XL7 is also exclusively nuclear (data not shown), consistent with at least one functional nuclear localization signal being located within the C terminus of the molecule. GFP-DmAos9 protein also mainly localizes within the nucleus (data not shown and Ref. 42).

Complex formation between DmUba2 and DmAos1 has important consequences for the subcellular localization of DmAos1. GFP-DmAos1, when expressed in COS cells, yielded a uniform (both cytoplasmic and nuclear) subcellular localization (Fig. 5B, panel 2). When 3-fold more pcDNA3-HA-DmUba2 (expressing an N-terminal HA-epitope tagged DmUba2) DNA was co-transfected, we could detect nuclear concentration of GFP-DmAos1 (Fig. 5B, panel 3). Co-transfection of 10-fold more pcDNA3-HA-DmUba2 (expressing an estimated 3-fold more HA-DmUba2 protein than GFP-DmAos1) could drive essentially all GFP-DmAos1 into the nucleus (Fig. 5B, panel 4), while the nuclear localization of GFP-DmUba2 did not change with co-transfection of 10-fold more pcDNA3-HA-DmAos1 (Fig. 5B, panel 5). This result suggests that upon complex formation, at least some DmUba2/DmAos1 heterodimers (possibly in the context of larger complexes with several other proteins (15, 45))
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Fig. 5. DmAos1 and DmUba2 can form a complex. A, His6-DmAos1 and GST-DmUba2 were coexpressed in E. coli strain BL21. Lysates were prepared as described under “Experimental Procedures.” Glutathione-Sepharose or Ni-NTA-agarose were used to pull down GST-DmAos1 and His6-DmUba2, respectively. Bound fractions were eluted with reduced glutathione (lanes 1 and 2) or imidazole (lanes 3 and 4). Aliquots of the eluates were treated with SDS sample buffer containing (lanes 2 and 4) or lacking (lanes 1 and 3) BME at 37 °C for 10 min, fractionated by SDS-PAGE, and stained with Coomassie Blue. The apparent large amount of GST-DmAos1 in lanes 1 and 2 were due to a much higher expression level of GST-DmAos1 when it was coexpressed with His6-DmAos1. Under both pull down conditions, a significant portion of His6-DmAos1 was in a p140 conformation, which returned to the p100 form upon BME treatment. Molecular weights (in kDa) are indicated on the gel. B, the subcellular localization of GFP-DmAos1 (lanes 2–4) in COS cells. Photos were taken 20 h after transfection. Right row: green fluorescence. Left row: phase contrast (except in panels 4 and 5, in which fluorescence is superimposed on phase contrast pictures). The constructs transfected into the cells were: panel 1, pEGFP-DmAos1; panel 2, pEGFP-DmAos1+ HA-DmAos1(3x); panel 3, pEGFP-DmAos1 + 3-fold excess of pcDNA3-HA-DmAos1; panel 4, pEGFP-DmAos1 + 10-fold excess of pcDNA3-HA-DmAos1; and panel 5, pEGFP-DmAos1 + 10-fold excess of pcDNA3-HA-DmAos1. GFP itself shows a uniform (both cytoplasmic and nuclear) distribution (data not shown). C, the expression levels of HA-DmAos1 and GFP-DmAos1 under the conditions in B were examined in an immunoblot probed with the anti-DmAos1 antiseraum that was raised against GST-XL7 (used at 1:1000 dilution). Lanes are numbered according to the panel numbers in B. Equal amount of total protein was loaded into each lane. The weak signal (of the same molecular weight as HA-DmAos1) present in lane 2 is the mammalian Uba2, which could be recognized by anti-DmAos1 antiseraum.

Fig. 6. DmAos1/DmAos1 can activate DmSUMO-1 in vitro. A, GST-K-DmSUMO-1 can form a thioester with a protein in fly head extract. Radioactively labeled GST-K-DmSUMO-1 (in reaction buffer containing 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM MgCl2, and 0.1 mM dithiothreitol) was incubated with fresh fly head extract (see “Experimental Procedures”) and/or ATP at 25 °C for 45 min. + and − above each lane indicate inclusion or exclusion of a component in a reaction. Reaction products were incubated at 37 °C for 10–15 min with SDS sample buffer containing (lanes 3 and 5) or lacking (lanes 1, 2, and 4) BME, subjected to SDS-PAGE and autoradiography. The bottom part of the gel containing free labeled GST-K-DmSUMO-1 (~40 kDa) was removed before autoradiography. Background is generally less when the samples are treated with BME before gel loading. Note the appearance of a novel signal (~170 kDa) in ATP and head extract dependent fashion (lane 4). This signal is sensitive to BME treatment (lane 5). B, same as in A except that purified recombinant His6-DmAos1/GST-DmAos1 dimer was used as the activating enzyme source. C, transfer of activated GST-K-DmSUMO-1 to GST-DmAos1. Conditions are similar to B except that purified recombinant GST-DmAos1 was included in all the reactions. A ~90-kDa signal appeared when labeled GST-K-DmSUMO-1, His6-DmAos1/GST-DmAos1, GST-DmAos1, and ATP were all present (lane 5). p90 is sensitive to BME treatment (lane 6). The ~120-kDa signal in lanes 5 and 6 is not sensitive to BME treatment.

In Vitro Evidence that DmAos1/DmAos1 Dimer Can Activate DmSUMO-1—A functional SUMO activating enzyme is expected to be able to form a thioester linkage with SUMO in an ATP-dependent fashion. To test the ability of DmAos1/DmAos1 to activate DmSUMO-1, we made a construct (pGEX-1KT-DmSUMO-1) for expressing in E. coli a GST fusion form of DmSUMO-1 (GST-K-DmSUMO-1) with a protein kinase A site inserted between the GST motif and DmSUMO-1 (DmSUMO-1 ending at Gly36). After purification, the protein was radioactively labeled with [γ-32P]ATP using the protein kinase A catalytic domain. When added to freshly prepared fly head extract, the labeled GST-K-DmSUMO-1 could induce the appearance of a novel band at 160–170-kDa position in an exogenous ATP-dependent manner (Fig. 6A, lane 4). This band is reducing agent sensitive (Fig. 6A, lane 5), suggesting it is a 120–130-kDa endogenous protein linked to labeled GST-K-DmSUMO-1 through a thioester bond. When labeled GST-K-
DmSUMO-1 was mixed with recombinant His6-DmAos1/GST-DmAos1 purified from E. coli, a similar band (with a slightly higher molecular weight than in the case of head extract, probably due to the presence of His-tag on His6-DmAos2) appeared only when ATP was present (Fig. 6B, lane 3). This band disappeared when the reaction product was treated with reducing agent (Fig. 6B, lane 4), suggesting the connection is also a thioester bond. The molecular mass of the labeled products (160–170 kDa) are higher than that which would be expected from the 95/100-kDa DmAos2 (endogenous head DmAos2 or His6-DmAos2) linked to GST-DmAos1. However, in the absence of reducing agent, a significant portion of purified His6-DmAos2 exists in a p140 form (see above, Fig. 5A). The ~170-kDa signal in the thioester formation assay using recombinant His6-DmAos2/GST-DmAos1 is likely the GST-K-DmAos1 modified conjugate of this p140 form. In the case of the head extract, it is possible that a proportion of endogenous DmAos2 exists in a form equivalent to p140 and it is this form that is most active in forming a thioester with DmSUMO-1.

We tested the ability of DmAos2/DmAos1 to transfer activated DmSUMO-1 to DmUb9. When purified recombinant GST-DmUb9 was included in the in vitro thioester formation assay, a 90-kDa (similar to the expected molecular mass for GST-K-DmSUMO-1 linked to GST-DmUb9) species appeared in a His6-DmAos2/GST-DmAos1 and ATP-dependent manner (Fig. 6C, lane 5). This species is also sensitive to reducing agent (Fig. 6C, lane 6), consistent with it being GST-DmUb9 linked to GST-K-DmAos1 through a thioester linkage. A 120-kDa species was also detected in the presence of the activating enzyme, GST-K-DmSUMO-1 and GST-Ubc9 (Fig. 6C, lane 5). This species is not sensitive to reducing agent (Fig. 6C, lane 6) and is possibly due to isopeptidyl bond linkage(s) between GST-K-DmSUMO-1 and GST-DmAos1 or between two or more GST-K-DmAos1 molecules. In a yeast two-hybrid assay, DmSUMO-1 interacts with DmAos1 (data not shown).

In Vivo Biochemical Evidence for DmAos2 Functioning as a DmSUMO-1 Activating Enzyme—To study the in vivo function of the Drosophila SUMO conjugating system, we created a putative dominant-negative mutant form of DmAos2, DmAos2(C175S). The yeast equivalent of this mutant, ScUba2(C177S), when expressed on a wild-type background, impaired host growth (15). P-element mediated germline transformation was used to generate fly lines that carry either a UAS-DmAos2 (wt) or UAS-DmAos2(C175S) cDNA insertion in their genome in a wild type genetic background. The GAL4/UAS technique (33) was employed to target overexpression of DmAos2 (wt) or DmAos2(C175S) protein in a tissue-specific manner. UAS-linked transgenes are not expressed unless a GAL4 activator that drives expression of UAS-linked genes.

We used C155 (39), a pan-neural GAL4 line to target expression of DmAos2 (wt) or DmAos2(C175S) to fly neurons. Immunoblotting analysis showed that the transgenes could indeed be induced to be overexpressed in adult head by C155 (Fig. 7A, compare lane 1 with lanes 2 and 3). In addition, we noticed a significant increase in DmSUMO-1 conjugate level in heads overexpressing DmAos2 (wt) (Fig. 7C, compare lane 1 with lane 2), consistent with DmAos2 being part of a DmSUMO-1 activating enzyme. Somewhat surprisingly, we did not observe a detectable decrease in DmSUMO-1 conjugate level in heads from flies overexpressing DmAos2(C175S); in fact, an increase was observed (Fig. 7C, compare lane 1 with lane 3; see “Discussion”).

In head extract from flies overexpressing DmAos2(C175S), the anti-DmAos2 antiserum could also recognize two minor, yet significant signals, p120 and 160 (Fig. 7A, lane 3). Overexpression of the immunoblot indicates that bands of the same sizes are also present in control (GAL4 alone; C155/Df(1)1w) or DmAos2 overexpressing head extracts, although at a much lower level (data not shown). Since the only difference between DmAos2 (wt) protein and DmAos2(C175S) protein is in the putative active site, we speculate that the difference in p120 and p160 signal intensity is caused by differences in the biochemical properties of cysteine and serine residues. Indeed, we found that the p120 species in DmAos2(C175S) head extract is sensitive to a brief NaOH treatment (Fig. 7B), while the corresponding band in DmAos2 (wt) head extract is not (data not shown), suggesting that the p120 species in the former is probably formed through an oxygen ester linkage between the substituted serine residue in DmAos2(C175S) and an endogenous protein with an apparent molecular mass of 20–25 kDa, while the corresponding p120 in the latter sample is formed.
This suggests it is not an active to NaOH or thiol treatment (Fig. 7). In contrast to p120, p160 in ability of the linkage in DmUba2(C175S) head extract are all protein or DmUba2(C175S) protein in p120 and the saponification level in DmUba2(wt) overexpresser head determine its NaOH sensitivity. However, the increase in Dm-
endogenous protein. The apparent higher level of p120 in DmUba2(C175S) head extract recognized by anti-DmSUMO-1 antiserum, one matches perfectly in its size with p120 (data not shown). DmUba2(C175S) head extract could be due to the fact that we treated our samples extensively with reducing agent in the process of making extracts, which would break most thioester linkages. Another factor that could contribute to p120 level difference is that the substituted serine in C175S mutant protein could form a more stable linkage with DmSUMO-1 and theoretically serve as a DmSUMO-1 trap, leading to more difference is that the substituted serine in C175S mutant protein being in a linked state than wild type DmUba2 even in live flies. Among the signals in DmUba2(C175S) head extract by anti-DmSUMO-1 antiserum, one matches perfectly in its size with p120 (data not shown). The weak intensity of this signal made it difficult to determine its NaOH sensitivity. However, the increase in Dm-SUMO-1 conjugate level in DmUba2(wt) overexpressing head extract, the size of the protein that is linked to DmUba2(wt) protein or DmUba2(C175S) protein in p120 and the saponifi-
cation of the linkage in DmUba2(C175S) head extract are all consistent with the notion that p120 is DmSUMO-1 linked to DmUba2(wt) or DmUba2(C175S). In contrast to p120, p160 in DmUba2(C175S) and DmUba2(wt) head extract is not sensitive to NaOH or thiol treatment (Fig. 7B and data not shown). This suggests it is not an in vivo correlate of the high molecular weight form of DmUba2 seen in bacteria, which is thiol sensitive. Currently we do not know the nature of the linkage in p160 (see “Discussion”).

The relative abundance of the SUMO conjugation components in Drosophila neurons (Fig. 3) and their potential interaction with CaMKII, which is enriched in fly neurons and important for synaptic plasticity (46), prompted us to look into the phenotypic consequences of overexpressing DmUba2(wt) and DmUba2(C175S) in the eye using GMR-GAL4 and more generally in Drosophila neurons using C155-GAL4. While most single P-element insertion lines driven by C155 (expressing an estimated 1–2-fold the endogenous level of DmUba2(wt) or DmUba2(C175S)) produced apparently normal adult progeny with no or very mild lethality at 29 °C (Table I and data not shown), two higher expression level lines (expressing an estimated 3–5-fold endogenous DmUba2 level equivalent of DmUba2(C175S)), C175S-117F and C175S-92E, yielded ~50% (Table I) and 100% lethality, respectively, in male progeny raised at 29 °C. Lethality for line C175S-92E was found to occur mainly at late pupal stage. Little lethality was observed at 25 °C (since GAL4 is cold sensitive) or in female progeny (since C155 is on the X chromosome and shows dosage compensation). Increasing the dosage of transgenes by putting both the

| UAS-Uba2 line | % Viability |
|---------------|------------|
| wt-53F        | 76         |
| wt-56E        | 54         |
| wt-56F        | 92         |
| wt-20B        | 81         |
| wt-49E        | 91         |
| wt-41B        | 98         |
| wt-49E + wt-41E | 16       |
| C175S-52F    | 60         |
| C175S-104E    | 56         |
| C175S-92E     | 0          |
| C175S-116E    | 54         |
| C175S-117F    | 43         |
| C175S-116E + C175S-117F | 3 |

Crosses and scoring are described under “Experimental Procedures.” One set of experimental results (including expression from double P-
elements and the corresponding single P-elements) is shown for each transgene.

Non-conserved Aspects of Yeast and Drosophila SUMO-1 Conjugation Systems—A couple of significant differences were seen between Drosophila and yeast SUMO-1 conjugation. A measurable portion of His6-DmUba2 (which has an N-terminal

C175S-117F and C175S-116E inserts into one line increases lethality to almost 100% (Table I) at 29 °C. Consistent with the lethality data, the expression level of DmUba2(C175S) in the double P-element line is comparable to that in C175S-92E (data not shown), suggesting a correlation between the severity of lethality and DmUba2(C175S) expression level. Similarly, increasing expression of DmUba2(wt) by using multiple P-
elements caused a more severe lethality phenotype than from either single P-element (Table I). Since in both DmUba2(wt)- and DmUba2(C175S)-overexpressing viable progeny, we detected an increase in DmSUMO-1 conjugate level in neurons (Fig. 7C), these lethality results suggest that increased DmSUMO-1 conjugation might be the cause of lethality when either DmUba2(wt) or DmUba2(C175S) was expressed to a sufficiently high level.

Limiting expression to the differentiated cells of the eye using GMR-GAL4 also produced a dose-dependent phenotype. Animals expressing high levels of DmUba2(C175S) (C155/Df (1)w; C175S-92E/+ ) had a rough, depigmented eye (Fig. 8B). GMR-GAL4 alone produces a slightly rough eye with normal pigmentation at 29 °C (Fig. 8A). Expression of lower levels of DmUba2(C175S) (line C175S-117E) produced a slightly depigmented eye, while expression of low levels of DmUba2(wt) (line wt-20B) did not produce a more severe phenotype than GMR-GAL4 alone (data not shown). In no case was the size of the eye reduced, suggesting that the depigmentation was due to degeneration of the retina as opposed to a failure of differentiation.

**DISCUSSION**

In this study, we describe the molecular identification of Drosophila Uba2, Aos1, Ubc9, and SUMO-1 homologs and the biochemical aspects of the DmSUMO-1 conjugation system. In addition, we found that at least one isoform of neuronal CaMKII is modified by DmSUMO-1. The relative abundance of the SUMO conjugation components in Drosophila neurons (Fig. 3) and their potential interaction with CaMKII, which is enriched in fly neurons and important for synaptic plasticity (46), prompted us to look into the phenotypic consequences of overexpressing DmUba2(wt) and DmUba2(C175S) in the eye using GMR-GAL4 and more generally in Drosophila neurons using C155-GAL4. While most single P-element insertion lines driven by C155 (expressing an estimated 1–2-fold the endogenous level of DmUba2(wt) or DmUba2(C175S)) produced apparently normal adult progeny with no or very mild lethality at 29 °C (Table I and data not shown), two higher expression level lines (expressing an estimated 3–5-fold endogenous DmUba2 level equivalent of DmUba2(C175S)), C175S-117F and C175S-92E, yielded ~50% (Table I) and 100% lethality, respectively, in male progeny raised at 29 °C. Lethality for line C175S-92E was found to occur mainly at late pupal stage. Little lethality was observed at 25 °C (since GAL4 is cold sensitive) or in female progeny (since C155 is on the X chromosome and shows dosage compensation). Increasing the dosage of transgenes by putting both the
His6-fusion) exists in a p140 form when purified from E. coli, while no such form was observed for the yeast His6-Uba2 (which had a C-terminal His6-fusion) (16). The importance of this form is suggested by the in vitro thioconjugation assays; the p140 form appears to be the active form of both the bacterially produced and endogenous fly head Uba2. A second difference we have noted is that a significant amount of DmSUMO-1 exists in unconjugated form in adult fly (Fig. 7C). This is also true for other developmental stages (data not shown). In contrast, yeast Smt3 seems to be completely in the conjugated form under steady state conditions (16). This pool of free SUMO-1 may be important in the mechanism(s) of lethality seen in flies with overexpressed DmUba2; lethality is associated with an increase in total SUMO-1 conjugates.

Overexpression of DmUba2 Is Lethal—in yeast, overexpression of ScUba2(wt) did not cause significant growth defects, while the ScUba2(C177A) and ScUba2(C177S) mutants did (15). The levels of SUMO-1 conjugates in these strains were not determined. When we overexpressed DmUba2(C175S) in fly head, we consistently observed the appearance of a NaOH-sensitive linkage formed between DmUba2(C175S) and an endogenous protein of about 20 kDa (presumably DmSUMO-1); no such linkage was observed for the equivalent yeast mutant protein ScUba2(C177S) when it was transiently overexpressed (15). Purified recombinant yeast ScUba2(C177S) mutant protein was not able to form any kind of linkage with Smt3 (16) in vitro. This difference could reflect a genuine activity difference in the two mutant proteins. Alternatively, the activities of DmUba2(C175S) and yeast ScUba2(C177S) could be impaired similarly and the short reaction time (10 min) allowed in the in vitro yeast experiments did not allow accumulation of sufficient yeast Uba2(C177S)-Smt3 species.

Overexpression of DmUba2(wt) protein in Drosophila neurons resulted in increased DmSUMO-1 conjugation in adult heads. This result is supportive of DmUba2 functioning to activate DmSUMO-1. Yeast ScUba2(C177S) was shown to impair host strain growth when overexpressed on a wild type background (15). The effect was previously explained as being due to the catalytically inactive mutant protein competing with ScUba2(wt) for binding partners and therefore interfering with ScUba2 function. Surprisingly, expression of DmUba2(C175S) did not lead to a detectable suppression in DmSUMO-1 conjugate level in progeny that survived to adulthood, rather an increase was detected (Fig. 7C). In DmUba2, the C175S mutation may not completely abolish the proteins ability to transfer DmSUMO-1 to DmUbc9. In our in vivo expression experiment, DmUba2(C175S) could function as a DmSUMO-1 trap (Fig. 7A), but the fact that not all of the mutant protein was SUMO-1-bound suggests that there is some turnover and transfer of SUMO-1. This is not unexpected, since there is precedent for activity of active site cysteine to serine mutations (48). At physiological pH, the nucleophilicity of a thiol would be expected to be approximately 1000-fold that of a hydroxyl group (49). Given the apparent partial activity of DmUba2(C175S), our results suggest that the nucleophilic addition is not the rate-limiting step in SUMO-1 transfer by DmUba2 or that the microenvironment of the catalytic site can reduce the difference between the reactivity of the thiol and the hydroxyl group.

Alternatively, the mutant protein could perform a function that is independent of its active site integrity and (possibly indirectly) contribute to DmSUMO-1 conjugate formation. Previously it was shown that the yeast Uba2 can interact with itself and other proteins (15, 45). Uba2 therefore can exist in multiple copies in a multicomponent complex and it is possible that one or more copies of Uba2 in these complexes perform a function other than direct catalysis, e.g. activating another copy of Uba2 to its full enzymatic activity. Yeast cells can tolerate a wide range (~10-fold) of variation in Smt3 conjugate level for normal growth rates (16). We observed that, for apparent normal development and survival, Drosophila can tolerate at least a 2-fold increase in DmSUMO-1 conjugate level (Fig. 7C). The lethality observed for lines that express very high levels of DmUba2(C175S) or DmUba2(wt) in the nervous system is probably due to a drastic increase in DmSUMO-1 conjugate level in the organism’s nervous system that is beyond tolerance. The degeneration of pigment cells and other retinal components is also likely due to an increase in conjugation.

A Novel in Vivo Modification Involving the DmUba2 Active Site—When DmUba2(C175S) was overexpressed in fly head, in addition to the p120 species, we also observed an increased level of another species, p160. In contrast to p120, p160 in DmUba2(C175S) progeny head extract is not sensitive to NaOH treatment (Fig. 7B), neither is p160 in DmUba2(wt) progeny head extract (data not shown), suggesting a covalent bond other than a thioester linkage is responsible for p160 in DmUba2(wt) overexpressor head extract. Elevated levels of p160 in DmUba2(C175S) head extract is not simply due to a higher level of total DmUba2 (wt + C175S) protein, since we did not detect a similar increase in p160 level in DmUba2(wt) progeny head extract containing an equivalent amount of total DmUba2 protein (Fig. 7A). Based on apparent molecular mass, the linkage in p160 is likely caused by a ~65-kDa moiety linked to the active site cysteine of DmUba2(wt) (or the substituted serine in C175S mutant protein) or some residue whose biochemical reactivity with or access to the moiety can be affected by the cysteine to serine substitution. Existence of p160, although at a low level, in control head extract confirmed the in vivo relevance of this modification. The identity of this novel modification and its potential function remain to be determined.

Interaction of DmUba2 and DmUbc9 with CaMKII—Both DmUba2 and DmUbc9 were initially identified in a yeast two-hybrid screen searching for Drosophila neuronal proteins that interact with CaMKII. However, upon secondary screening with independent in vitro assays (coimmunoprecipitation and GST fusion protein pull down) for protein-protein interactions, neither DmUba2 nor DmUbc9 interacted stably with CaMKII. Our finding that at least one isoform of neuronal CaMKII is modified by DmSUMO-1 strongly suggests that the interactions observed in the yeast two-hybrid screen normally contribute to catalysis. Since the modification site on CaMKII is not mapped, currently it is not known whether the two-hybrid interaction between CaMKII and DmUbc9 involves regions close to where catalysis happens or remote regions that aid in transient catalytic complex formation. The finding that DmUba2 can also interact with CaMKII suggests the interesting possibility that during the modification of CaMKII by DmSUMO-1, a multicomponent complex (at least consisting of the DmSUMO-1 activating and conjugating enzymes) transiently form around CaMKII.

From the identified functions of SUMO modification in other species, DmSUMO-1 modification could potentially change the subcellular localization of CaMKII (by creating or exposing a binding site), protect CaMKII from degradation (by competing with a ubiquitination site), or modulate CaMKII activity (by interfering with the function of the catalytic domain or regulatory region). Recently, it has been reported that the nuclear protein kinase HIPK2 is modified by SUMO-1 and that this modification changes the localization of HIPK2 within the nucleus and makes it associate with an insoluble nuclear fraction (50). To date, no Drosophila CaMKII isoforms containing nuclear localization signals have been cloned, but isolated adult
head nuclei contain insoluble, 50–60-kDa forms of the kinase as determined by immunoblotting. The CaMKII isoform that is modified in vivo by DmSUMO-1 remains to be identified.

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