To identify proteins that regulate the function of Dorsal, a Drosophila Rel family transcription factor, we employed a yeast two-hybrid screen to search for genes encoding Dorsal-interacting proteins. Six genes were identified, including two that encode previously known Dorsal-interacting proteins (Twist and Cactus), three that encode novel proteins, and one that encodes Drosophila Ubc9 (DmUbc9), a protein thought to conjugate the ubiquitin-like polypeptide Smt3 to protein substrates. We have found that DmUbc9 binds and conjugates Drosophila Smt3 (DmSmt3) to Dorsal. In cultured cells, DmUbc9 was found to relieve inhibition of Dorsal nuclear uptake by Cactus, allowing Dorsal to enter the nucleus and activate transcription. The effect of DmUbc9 on Dorsal activity was potentiated by the over-expression of DmSmt3. We have also identified a DmSmt3-activating enzyme, DmSAE1/DmSAE2 and found that it further potentiates Dorsal-mediated activation.

A very similar pathway involving the vertebrate homolog of Cactus, IxB, is involved in the regulated nuclear import of vertebrate Rel family proteins, such as NFκB (9, 10). In addition, recent studies suggest that the nuclear import of NFκB may be influenced by the Smt3 conjugation pathway (11). Smt3 is a small ubiquitin-like protein that can be enzymatically conjugated to various protein substrates via an amide linkage between the C-terminal carboxyl group of Smt3 and a lysine ε-amino group on the target protein. Conjugation of mammalian SMT3C to IxB is thought to stabilize IxB by blocking ubiquitylation and therefore subsequent proteasomal degradation. By stabilizing IxB, the vertebrate Smt3 conjugation pathway results in the down-regulation of NFκB activity.

In an effort to illuminate further the mechanisms by which Dorsal activity is regulated, we sought to identify novel Dorsal interacting proteins via a yeast two-hybrid screen. One of the proteins identified in this screen was Drosophila Ubc9 (DmUbc9) (12). This protein is homologous to yeast and mammalian Ubc9, which are thought to function as Smt3-conjugating enzymes (13–16). Experiments examining the effect of DmUbc9 on Dorsal nuclear uptake and Dorsal-mediated transcriptional activation demonstrate that the effect of the Smt3 conjugation pathway on Dorsal activity is opposite to the effect of this pathway on the activity of the vertebrate Rel family protein NFκB. In particular, we find that DmUbc9 conjugates Drosophila Smt3 (DmSmt3) to Dorsal and overcomes Cactus-dependent sequestration of Dorsal in the cytoplasm. Furthermore, we find that the effects of DmUbc9 on Dorsal activity are enhanced by overexpression of DmSmt3 and a DmSmt3-activating enzyme. Thus, the Smt3 conjugation pathway enhances Dorsal nuclear translocation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—For Dorsal deletions fused to the LexA DNA-binding domain, dorsal cDNA was amplified using PCR1 primers containing Smal (5) and Sall (3) restriction sites. dorsal inserts were ligated to a Sall/SalI-digested modified LexA vector constructed by inserting the Sp61 cassette from pBTM116 into the LexA selectable vector pGAD424 at its SphI sites (17). Total RNA was isolated from 0–3-h-old embryos using the manufacturer’s protocol for Trizol reagent (Life Technologies, Inc.). Purification of Poly(A)1 RNA was performed using an oligo(dT) column from Collaborative Biomedical Products as per their protocol. Synthesis of double-stranded cDNA from mRNA was performed using the SuperScript Plasmid System and its accompanying protocol from Life Technologies, Inc. The resulting cDNA contained SalI and NotI linkers that were ligated to the G418 transactivation domain-containing vector pUC86 at the SalI and NotI sites (18).

The yeast strain L40, which contains integrated HIS3 and lacZ reporters under the control of a GAL1 promoter downstream from four LexA binding sites, was transformed with the Dorsal-LexA fusion vec-
SmaI primers containing ORF using the TNT system (Promega). pGem-dl was generated by BamHI digestion of X-gal vector and a fragment containing the full-length ORF was inserted into the pPac vector. Expression vectors pPac-dl and pPac-dl-HA, pPac-DmUbc9, and the cDNA library were prepared from pGem (Promega) containing GST-Su(H) fusion proteins. Plasmids were mini-prepped using glass beads as described (19) and retransformed into the trp+ strain of Escherichia coli (K8) via electroporation to rescue the library containing plasmid, which carried a trp+ marker. Plasmids from K8 cultures were then mini-prepped and sequenced.

Yeast Two-hybrid Screen—The expression vectors pPac-DmUbc9 and pPac-HA-cactus were constructed by amplifying cDNA encoding DmUbc9 or Cactus with PCR primers containing KpnI sites and inserting each PCR product into the Kpn1 site in the pPac-HA vector (47). The pPac-HA-DmSAE2 vector was made by amplifying the cDNA encoding DmSAE2 or DmSm3 by inserting the PCR product containing Smal sites and inserting it into the Kpn1 site in the pPac-HA vector. The pPac-HA-DmSm3 vector was made by amplifying the cDNA encoding DmSm3 and inserting it into the Kpn1 site in the pPac-HA vector. The pPac-HA-DmSm3 vector was made by amplifying the cDNA encoding DmSAE2 or DmSm3 from the EST clone L025277 or “cDNA clone 240” (a generous gift from A. K. Hardin), respectively, using primers containing KpnI and SacI sites (3’ and 5’) and inserting each into the Kpn1 and SacI sites in the pPac-HA vector. All EST clones were generated by the Berkeley Drosophila Genome Project and purchased from Research Genetics Inc. Lastly, the pPac-FLAG-Dorsal expression vector was constructed by amplifying the full-length dorsal ORF using PCR primers containing Kpn1 sites and inserting it into the pPac-M2 vector (47). The indicated combinations of these vectors (4 μg each) were brought to 20 μg total with empty vector and co-transfected into S2 cells by the calcium phosphate method as described (21). After approximately 48 h, cells were washed once in phosphate-buffered saline and lysed either directly in SDS-PAGE buffer (5% SDS, 0.15M Tris-HCl (pH 6.7) 30% glycerol) or in Strong Lysis Buffer (CytoSignal, Inc.). For immunoprecipitation experiments, the Strong Lysis Buffer lysate was incubated with anti-M2 beads (Sigma) overnight at 4°C; the beads were then washed twice with ice-cold Strong Lysis Buffer and taken up in SDS-PAGE loading buffer. For Western blot analysis, SDS-PAGE sample buffer lysates were fractionated by 8% SDS-PAGE. For immunoprecipitations, the beads were vortexed, boiled, and spun down, and the supernatants were fractionated via 15% SDS-PAGE. In both cases, proteins were transferred to polyvinylidene difluoride membranes and detected by ECL according to the manufacturer’s recommendations (Roche Molecular Biochemicals). Primary antibodies for the Western blots in Figs. 4 and 5 were anti-FLAG M2 (Sigma, F3165) and anti-HA 12CA5 (Roche Molecular Biochemicals). We also used anti-HA 3F10 with similar results (data not shown).

RESULTS

Yeast Two-hybrid Screen—A yeast two-hybrid screen was employed to identify proteins that interact with Dorsal. Because full-length Dorsal was able to activate transcription in yeast on its own, a deletion analysis was carried out to define the longest form of Dorsal unable to activate transcription in yeast (Fig. 1). Consistent with previous studies (22, 25), we found that Dorsal contains a C-terminal domain required for efficient activation, because deletion of 203 residues from the C-terminus abolished activity. Although the C-terminal domain is required for activation, the N-terminal region of Dorsal (which includes the Rel homology domain) is thought to be sufficient for many of the other functions of this factor, including regulated nuclear uptake and transcriptional repression (22, 25, 26). Thus, this N-terminal region, although not sufficient for activation, must mediate many functionally relevant protein interactions (Promega). We therefore decided to explore a chimeric protein consisting of the LexA DNA-binding domain fused to the N-terminal 470 amino acids of Dorsal to screen an expression library for genes encoding Dorsal-interacting proteins.

We screened a Drosophila 0–3-h embryonic cDNA library designed to produce Gal4 activation domain fusion proteins. In
a screen of $1.4 \times 10^6$ cDNAs, we obtained 306 positive clones encoding proteins that could interact with the LexA-Dorsal-470 fusion protein to trigger expression of HIS3 and lacZ reporters. Because we were only interested in cDNAs encoding proteins that interact with Dorsal in a specific manner, we used the yeast two-hybrid system to test these clones for their ability to interact with LexA-Bicoid, LexA-Groucho, and LexA-E(spl)m7, in addition to LexA-Dorsal-470. All of these LexA fusion proteins have been successfully employed in yeast two-hybrid screens or assays, indicating that they are all expressed in yeast cells (data not shown). 165 of the clones were found to encode proteins that interacted well with all of the LexA fusion proteins tested. These were deemed to be nonspecific interactors and were eliminated from further consideration. The 141 remaining clones encoded proteins that interacted solely or primarily with the LexA-Dorsal-470 fusion protein.

Restriction and sequence analysis of the 141 specifically interacting isolates showed that they defined just six different genes, which we initially termed dip1 through dip6. Dip1, Dip3, Dip4, Dip5, and Dip6 interact strongly with LexA-Dorsal-470 and not at all with LexA-Bicoid, LexA-Groucho or LexA-E(spl)m7, whereas Dip2 interacts strongly with LexA-Dorsal-470, very weakly with LexA-Groucho, and not at all with LexA-Bicoid or LexA-E(spl)m7 (see “Experimental Procedures” for further details).

Sequence analysis revealed that dip6 is cactus, which encodes a cytoplasmic inhibitor of Dorsal (27–30), and that dip5 is twist, which encodes a protein known to interact with Dorsal to synergistically activate the transcription of specific dorsoventral patterning genes (22, 31, 32). Thus, two of the six genes isolated in the screen were previously known biologically relevant Dorsal-interacting proteins. A third gene, dip4, was found to encode DmUbc9 (12), a homolog of yeast and human Ubc9. The remaining three clones, dip1, dip2, and dip3, encode novel factors and will be further described elsewhere. Their sequences have been deposited in the GenBankTM data base. Northern analysis of poly(A)⁺ RNA and in situ hybridization experiments indicate that DmUbc9 mRNA is probably provided maternally and is uniformly expressed throughout embryogenesis (data not shown).

**DmUbc9 Interacts with Dorsal and Cactus in Vitro**—To confirm that the observed interaction between Dorsal and DmUbc9 was direct, a GST-DmUbc9 fusion protein was immobilized on glutathione beads and assessed for its ability to retain in vitro translated Dorsal. Consistent with the yeast two-hybrid results, GST-DmUbc9 binds Dorsal (Fig. 2B, lane 7). The specificity of the interaction is demonstrated by the absence of interactions between Dorsal and GST (lane 4), Dorsal and an unrelated GST fusion protein (lane 10), or an unrelated in vitro translated protein and GST-DmUbc9 (lane 9). Because two other groups had demonstrated interactions between mammalian Ubc9 and IκB (11, 33), we reasoned that the Drosophila homolog of IκB, Cactus, might also interact with DmUbc9, and we thus carried out a similar assay to examine binding of DmUbc9 to Cactus. Cactus does indeed bind GST-DmUbc9 (lane 8), but not GST (lane 5) or the unrelated GST fusion protein (lane 11).

**DmUbc9 Facilitates Nuclear Import of a Dorsal-GFP Fusion Protein**—Inhibition of Dorsal activity by Cactus involves retention of Dorsal in the cytoplasm. Our finding that DmUbc9 interacts with both Dorsal and Cactus suggested that DmUbc9 might influence Dorsal nuclear localization. To visualize effects of DmUbc9 on the nuclear uptake of Dorsal, we constructed a Dorsal-GFP fusion protein. Because regions in the N-terminal half of Dorsal have been implicated in its regulated nuclear uptake, GFP was fused to the C terminus of Dorsal (26). The chimeric polypeptide was expressed in S2 cells with or without Cactus and DmUbc9. Dorsal-GFP predominantly localizes to the nucleus when expressed alone (Fig. 3, top row). This is in accord with previous studies demonstrating that transiently expressed Dorsal protein localizes to the nuclei of S2 cells (34). Although these cells do contain endogenous Cactus protein, the level of Dorsal or Dorsal-GFP expression obtained in the transient transfection experiments is apparently high enough to overwhelm the ability of endogenous Cactus to retain Dorsal in the cytoplasm. Support for this idea comes from the finding that inclusion of an expression vector encoding Cactus in the transient transfection assay results in Dorsal-GFP being al-

![Fig. 1. Mapping of the Dorsal activation domain using the yeast two-hybrid assay.](Image)

![Fig. 2. DmUbc9 interacts with Dorsal and Cactus in vitro. A, GST (lane 1), GST-DmUbc9 (lane 2), and GST-Su(H) (lane 3) were expressed in E. coli, purified on glutathione-agarose beads, and analyzed by 12% SDS-PAGE followed by staining with Coomassie Blue. B, in vitro translated ³⁵S-labeled Dorsal (lanes 4, 7, and 10), Cactus (lanes 5, 8, and 11), or luciferase (lanes 6, 9, and 12) were incubated with equal amounts of the GST (lanes 4–6), GST-DmUbc9 (lanes 7–9), or GST-Su(H) (lanes 10–12). The beads were then washed several times, eluted with sample buffer, and then subjected to SDS-PAGE and autoradiography. Lanes 1–3 show an amount of Dorsal (lane 1), Cactus (lane 2), or luciferase (lane 3) input protein equal to 20% of that used in the assays shown in lanes 4–12.](Image)
most completely retained in the cytoplasm (Fig. 3, middle row). Introduction of DmUbc9, however, reverses the effect of Cactus on Dorsal-GFP localization (Fig. 3, bottom row). In contrast, Cactus and DmUbc9 have no effect on the subcellular localization of untagged GFP (data not shown). These results demonstrate that DmUbc9 can overcome the Cactus-mediated inhibition of Dorsal nuclear uptake.

To further explore the functional significance of the Cactus-DmUbc9-Dorsal interaction, we performed a transient transfection assay based on an approach utilized previously (22). The Dorsal-responsive reporter employed in this assay consisted of five tandemly repeating Dorsal and Twist binding sites driving expression of a luciferase reporter (Fig. 4). Twist and Dorsal are known to activate this promoter synergistically in embryos as well as in cell culture (22, 32). The reporter was co-transfected into S2 cells along with expression vectors for Dorsal, Twist, Cactus, and/or DmUbc9. Co-expression of Twist and Dorsal enhanced reporter activity roughly 8-fold, whereas addition of Cactus resulted in a reduction in activity back to the basal level (Fig. 4B). Inhibition of Dorsal-dependent reporter activity by Cactus was mitigated in a dose-dependent manner by co-expression of DmUbc9, consistent with the idea that DmUbc9 overcomes Cactus-mediated sequestration of Dorsal in the cytoplasm.

To determine whether the effect of DmUbc9 on Dorsal nuclear uptake and Dorsal activity reflects the formation of a complex between DmUbc9, Dorsal, and/or Cactus, cDNAs encoding DmUbc9 and Cactus were fused to sequences encoding an N-terminal HA-tag and different combinations of these fusion proteins were co-expressed with FLAG-tagged Dorsal in S2 cells. The cells were then lysed under conditions that disrupt low affinity but not high affinity protein-protein interactions. FLAG-tagged Dorsal and any associated proteins were precipitated with agarose beads conjugated to anti-FLAG antibody and analyzed by Western blotting. The name “Ubc9” reflects the homology of this protein to ubiquitin-conjugating enzymes. However, recent studies on yeast and human Ubc9 have shown that this enzyme primarily conjugates the yeast protein Smt3p or its human homologs SMT3A, SMT3B, and SMT3C rather than ubiquitin to proteins (13–16). We therefore were interested in assessing the effect of Smt3 on Dorsal-dependent transcriptional activation. For these purposes, we employed a previously reported Drosophila cDNA encoding a protein (which we term DmSmt3) that shares greater than 70% identity with human SMT3A and SMT3B and roughly 50% identity with yeast Smt3p and human SMT3C (Fig. 5A) (35). This may represent the only Smt3 family protein in Drosophila, because a search of the Drosophila Genome Project data base including the EST data base did not reveal any other potential homologs. However, we cannot at this point definitively rule out the existence of other Smt3 family members in Drosophila. DmSmt3 was found to stimulate reporter gene activity to a similar extent as DmUbc9 (Fig. 5B). Co-transfection of vectors encoding both DmUbc9 and DmSmt3 led to a greater than additive stimulation of reporter activity. These data suggest that DmUbc9 and DmSmt3 function together to facilitate Dorsal nuclear uptake.

Because Smt3 conjugation has never been demonstrated in Drosophila, we wished to confirm that Ubc9 is indeed an Smt3-conjugating enzyme in vivo and to determine whether Dorsal is a target for Smt3 conjugation. Thus, different combinations of HA-tagged DmUbc9, HA-tagged DmSmt3, and HA-tagged Cactus were co-expressed with FLAG-tagged Dorsal in S2 cells. Cells were then lysed in SDS-PAGE loading buffer, and the resulting whole cell lysates were fractionated by SDS-PAGE and analyzed by Western blotting. The anti-HA Western blot revealed a series of high molecular polypeptides, which were only detected in cells that had been transfected with HA-tagged DmSmt3 (Fig. 5C, upper panel). The appearance of these bands was greatly enhanced by the presence of HA-tagged DmUbc9 (compare lanes 4 and 5). Overexpression of Smt3 in yeast results in a similar array of high molecular mass Smt3-conjugated proteins (16). In addition to the high molecular mass
bands, a DmSmt3/DmUbc9-dependent band with an apparent molecular mass equal to that expected for an Smt3-Dorsal conjugate was also visible in the anti-HA Western blot. A Western blot using antibodies against the FLAG-tagged Dorsal protein (Fig. 5C, lower panel) confirms that this band represents Smt3-conjugated Dorsal. Fold activation values are obtained by dividing each relative luminescence value by the relative luminescence value obtained for the reporters alone. C, co-immunoprecipitation assays on extracts of transfected S2 cells. S2 cells were co-transfected with expression vectors (4 μg each) encoding the indicated proteins and lysed in Strong Lysis Buffer (CytoSignal) 48 h later. Lysates were then immunoprecipitated overnight with anti-M2 beads (Sigma). Proteins were fractionated by 15% SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Asterisks indicate bands corresponding to the anti-M2 antibody eluted from agarose beads used in the immunoprecipitation. The positions of molecular mass standards are indicated in kDa at the left.

**FIG. 5.** Dorsal is a substrate for DmSmt3 conjugation. A, DmSmt3 shares 75, 77, and 55% identity with human SMT3A, SMT3B and SMT3C, respectively, and 52% identity with *Saccharomyces cerevisiae* Smt3p. B, the DE5–37tkluc reporter was co-transfected with expression vectors for the indicated proteins. Data were analyzed as described in the legend to Fig. 4B. C, S2 cells were co-transfected with expression vectors (4 μg each) encoding the indicated proteins and lysed in SDS-PAGE buffer 48 h later. Proteins were fractionated by 8% SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Asterisks indicate Drosophila proteins that cross-react with the anti-HA antibody. The positions of molecular mass standards are indicated in kDa at the left.
Smt3-activating enzymes and assess them for enhancement of reporter activation. A search of the Berkeley Drosophila Genome Project EST data base revealed a single cDNA with high homology to SAE1 and a single cDNA with high homology to SAE2. Full-length cDNAs were obtained from the genome project and sequenced. The SAE1 homologous clone (DmSAE1) was found to encode a 337-amino acid protein displaying 39% identity with human SAE1 and 27% identity with \textit{S. cerevisiae} Aos1p (ScAos1p) (16, 37). The SAE2 homologous clone (DmSAE2) encoded a 700-amino acid protein that displays 46% identity with human SAE2 (HsSAE2) and 29% identity with \textit{S. cerevisiae} Uba2p (ScUba2p) (16, 37).

To examine the effect of DmSAE1/DmSAE2 on Dorsal-dependent reporter activation, sequences encoding each protein were cloned into an expression vector. These vectors were introduced into S2 cells along with the Dorsal-responsive reporter and combinations of expression vectors for DmSmt3, DmUbc9, Cactus, Dorsal, and Twist. Although DmSAE1/DmSAE2 alone was able to alleviate Cactus inhibition of reporter activity to a small degree, co-expression of the activating enzyme with DmSmt3 and DmUbc9 led to a more than additive increase in reporter activity (Fig. 6C). These findings provide additional support for the idea that Smt3 conjugation enhances Dorsal activity.

**DISCUSSION**

In this study, we demonstrate that DmUbc9, an Smt3-conjugating enzyme (13–16), is a Dorsal-interacting protein. We show that DmUbc9 catalyzes the conjugation of DmSmt3 to Dorsal and opposes the inhibitory effect of Cactus on Dorsal nuclear uptake. We also report the identification of DmSAE1/DmSAE2, the \textit{Drosophila} homolog of the Smt3-activating enzyme. We further show that DmSmt3, DmSAE1/DmSAE2, and DmUbc9 function synergistically to stimulate the activity of a Dorsal-responsive reporter gene. These findings suggest that the Smt3 conjugation pathway enhances Dorsal activity by enhancing its nuclear uptake. Although Ubc9 family proteins have been found to interact with a variety of transcription factors (33, 38, 39), to our knowledge, this is the first study to show that Ubc9 can actually conjugate Smt3 to a sequence-specific transcription factor and enhance its activity. Furthermore, our finding that the effects of DmUbc9 on Dorsal activity are potentiated by DmSmt3 and a DmSmt3-activating enzyme provides some of the best evidence to date that Smt3 conjugation is directly involved in regulating transcription factor activity.

**Smt3 Conjugation as an Evolutionarily Conserved Pathway for the Regulation of Subcellular Localization—Smt3 homologs**
have been cloned from eukaryotes as diverse as yeast, Arabidopsis, and humans (16, 35, 40, 41). These proteins in general display greater than 50% identity with one another but also roughly 20% identity with ubiquitin. The identification of the components of the Smt3 conjugation pathway in yeast, humans, and now Drosophila has revealed that Smt3 conjugation and ubiquitin conjugation proceed by similar pathways (16, 37, 42). Both pathways require an activating enzyme, or E1 protein, which becomes covalently attached to ubiquitin or Smt3 via a high energy thioester bond, and a conjugating enzyme, or E2 protein, which accepts ubiquitin or Smt3 from the E1 protein forming a second thioester-linked covalent complex. Ubiquitin or Smt3 is then transferred to an E2 protein group on a final protein substrate. The transfer of ubiquitin from the E2 protein to the final substrate often requires a ubiquitin ligase, or E3 protein. In contrast, an E3-type protein is apparently not required for Smt3 conjugation. Although ubiquitin conjugation targets proteins for proteasomal degradation (42), Smt3 conjugation appears to serve other purposes. Originally identified in yeast as an enzyme required for proper cell cycle progression, Ubc9 has been found to physically interact with a diverse array of proteins including RanGAP1, PML (promyelocytic leukemia protein), bleomycin hydrolase, E2A, androgen receptor, and c-Rel (11, 33, 38, 39, 43, 44). Association of human Ubc9 with RanGAP1 results in the conjugation of RanGAP1 to the Smt3 homolog SMT3C/SUMO-1 (small ubiquitin-related modifier), allowing it to bind RanBP2 at the nuclear periphery. This allows RanGAP1 to stimulate GTP hydrolysis by Ran. Only SMO-1-conjugated RanGAP1 binds to RanBP2, implying that SMT3C and Ubc9 are required for nuclear import. In the case of PML, interaction with Ubc9 and subsequent SMO-1 conjugation is essential for targeting PML to discrete subnuclear structures known as PML-bodies or nuclear dots. In acute promyelocytic leukemia cells, the subnuclear localization of PML is altered, suggesting that improper SMO-1 conjugation may trigger oncogenesis. These studies argue that one function of Smt3 conjugation is to regulate the subcellular localization of proteins.

Roles for Smt3 Conjugation in the Regulation of Rel Family Protein Activity—Although we have found a possible role for Smt3 conjugation in regulating Dorsal activity, a number of reports have implicated Ubc9 in the modulation of transcriptional activation by other Rel family proteins. For example, a recent study showed that SUMO-1-conjugated IκB is resistant to degradation and, accordingly, that SUMO-1 and Ubc9 work together to inhibit activation of an NFκB-dependent reporter (11). This contrasts with our findings showing that the Smt3 conjugation pathway activates Dorsal-dependent reporters. This difference could relate to inherent differences between the NFκB/IκB and Dorsal/Cactus pathways. However, an earlier report (33) suggests that mammalian Ubc9 can enhance Rel protein function via an interaction with NFκB and/or IκB. Thus, an alternative explanation for the different effects of Smt3 conjugation on Rel protein activity could be that different Smt3 family proteins have different functions. An alignment of DmSmt3 with the three members of the human Smt3 family (Fig. 5A) reveals that DmSmt3 displays significantly higher homology to SMT3A and SMT3B (77 and 75%, respectively) than to SMT3C/SUMO-1 (55%). Thus, DmSmt3, SMT3A, and SMT3B appear to define an Smt3 subfamily that is distinct from SMT3C/SUMO-1. Perhaps SMT3C/SUMO-1 antagonizes transcriptional activation by Rel proteins, whereas SMT3A/B-like proteins (such as DmSmt3) enhance Rel protein function.

The Smt3 conjugation system may also function at other levels in the regulation of Rel family protein activity. For example, Ubc9 has been shown to associate with the type I TNFα receptor and MEKK1 and to synergize with MEKK1 to activate an NFκB-dependent reporter (45).

Although we were able to detect a DmSmt3-Dorsal conjugate in cells that were simultaneously co-transfected with Dorsal, DmUbc9, and DmSmt3, the level of conjugation was low: no more than about 10% of the Dorsal protein was found in the DmSmt3-conjugated form. This contrasts with the results of our experiments looking at the localization of a Dorsal-GFP fusion protein, in which we found that DmUbc9 was able to direct the relocalization of a large fraction of the Dorsal-GFP in these cells from the cytoplasm to the nucleus. This suggests that the conjugation of DmSmt3 to Dorsal may be transient. Perhaps Dorsal and DmSmt3 are deconjugated as soon as Dorsal enters the nucleus. In accord with this idea, recent observations suggest that a dynamic equilibrium may exist between Smt3-conjugated and unconjugated protein species. In yeast, the vast majority of cellular Smt3p is conjugated to other proteins, although the population of proteins that is covalently modified changes during the cell cycle. Furthermore, a yeast enzyme capable of catalyzing the deconjugation reaction has been identified, and homologs of this enzyme appear to exist in many other eukaryotic species (46).

A genetically defined locus, termed semushi, was recently found to be identical with DmUbc9. Experiments employing the semushi allele suggest that DmUbc9 may be necessary for the nuclear import of the anteroposterior patterning morphogen Bicoid (24). This study was silent about potential roles of the Smt3 conjugation pathway in other developmental processes. However, a recent preliminary analysis of embryos lacking maternally supplied DmUbc9 indicates the presence of multiple patterning defects of varying penetrance. Because of the complex nature of these defects, their characterization will require extensive phenotypic analysis and the generation of additional DmUbc9 alleles. The possibility that DmUbc9 has pleiotropic developmental roles is not surprising given increasing evidence for wide spread roles of Smt3 conjugation in transcription factor function and in the targeting of proteins to their proper subcellular locales.

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Note Added in Proof—Since the submission of this paper, Smt3/SUMO-1 modification has been reported to activate the transcriptional response of p53 (Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. (1999) EMBO J. 18, 6462–6471; Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999) EMBO J. 18, 6455–6461).

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