dSmurf Selectively Decays Decapentaplegic-activated MAD, and Its Overexpression Disrupts Imaginal Disc Development*

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MAD plays an important role in decapentaplegic (DPP) signaling throughout Drosophila development. Despite a recent study describing the restriction of DPP signaling via putative ubiquitin E3 ligase dSmurf (1), the molecular mechanisms of how dSmurf affects DPP signaling remain unexplored. Toward this goal we demonstrated the degradation of phosphorylated MAD by dSmurf. dSmurf selectively interacted with MAD, but not Medea and Dad, and the MAD-dSmurf interaction was induced by constitutively active DPP type I receptor thickveins. Wild type dSmurf, but not its C1029A mutant, mediated ubiquitination-dependent degradation of MAD. Silencing of dSmurf using RNA interference stabilized MAD protein in Drosophila S2 cells. Targeted expression of dSmurf in various tissues abolished phosphorylated MAD and disrupted patterning and growth. In contrast, similar overexpression of inactive dSmurf(C1029A) showed no significant effects on development. We conclude that dSmurf specifically targets phosphorylated MAD to proteasome-dependent degradation and regulates DPP signaling during development.

Decapentaplegic (DPP)1 is the Drosophila ortholog of human bone morphogenetic protein (BMP) 2/4, which belongs to the transforming growth factor-β (TGF-β) superfamily (2–4). DPP plays a central role in Drosophila morphogenesis. DPP signals through cell surface receptors and intracellular Smad proteins. In Drosophila, all three classes of Smads, R-Smad (MAD, dSmad2), Co-Smad (Medea), and I-Smad (Dad), have been characterized (2). MAD, the founding member of Smad family (5), transduces the signal of DPP, while dSmad2 mediates TGF-β/Activin signaling (2).

Ubiquitination is catalyzed by an enzymatic cascade including ubiquitin-activating E1, ubiquitin-conjugating E2, and specific ubiquitin E3 ligase. The specificity of ubiquitination derives from the highly specific protein-protein interaction between E3 ligase and substrate (6, 7). In TGF-β signaling, HECT-class E3 ligases Smurf1 and Smurf2 specifically promote degradation of Smad1, Smad2 (5–7) and possibly I-Smads and TGF-β type I receptor (11, 12). In Xenopus laevis, Smurfs showed inhibition of BMP signaling and affected pattern formation (8). Recently, a Drosophila ligase E3 dSmurf has been identified by genetic screen (1); the null mutation of dSmurf resulted in a lethal defect in hindgut organogenesis. However, the mechanisms underlying dSmurf actions in DPP signaling and embryogenesis remain unclear.

In this study, we present biochemical and genetic evidence for the biological functions of dSmurf. The results revealed that dSmurf specifically inhibited DPP signaling by targeting DPP-activated MAD, not dSmad2, Medea, or Dad, for ubiquitination/proteasome-dependent proteolysis. Targeted overexpression of dSmurf in imaginal discs strongly reduces the level of phosphorylated MAD and consequently causes disruption of DPP-dependent patterning of imaginal discs, demonstrating that dSmurf is a negative regulator of DPP signaling in vivo.

EXPERIMENTAL PROCEDURES

Molecular Cloning—dSmurf were obtained by using PCR with primers (forward: gGAATTC aat aat gag tac cca cca and reverse: ttcGTC-GAC tta etc ccc ggc aac tcc aca) from total fly cDNA (Clontech) or Lack cDNA clone (gift of Scott Hawley), and cloned into the EcoRI and SalI of pXF1 or pXFP1M, for making FLAG-tagged or Myc-tagged dSmurf, respectively. His-tagged MAD is made by subcloning into a mammalian expression vector pXPF2RH (9). The point mutation of dSmurf of C1029A substitution was generated from two-step PCR. MAD(28A) mutant was made by mutating the two Ser into Ala in the SVS phosphorylation motif.

 Yeast Two-hybrid Assay—A LexA-based yeast two-hybrid assay was performed as described previously (9). MAD, dSmad2, and Dad were obtained by PCR from respective cDNA (gifts of Kohei Miyazono and Richard Padgett) and cloned into a yeast bait vector pEG202. dSmurf wild type and the dSmurf deletion mutants, obtained by PCR, were cloned into prey vector pJoG-45 at sites of EcoRI and SalI. Smurf1 (gift of Jeff Wray) was also subcloned into pJoG-45. β-Galactosidase activity is measured by liquid assay according to the method of Matchmaker two-hybrid system 2 (Clontech). Immunoprecipitation and Western Blot—Culture and transfection of human kidney 293T cells and Drosophila Schneider S2 cells, and coupled immunoprecipitation-Western blot assays were carried out as described previously (13, 14).

For degradation assay, copper-inducible pMK33 expression plasmids for MAD, dSmurf, and the constitutively active type I receptor Thickveins (aTKV) (gifts of Richard Padgett) were transfected into Schneider S2 cells by calcium phosphate. In the case of RNA interference experiment (Fig. 4D), cells were cotransfected with synthetic 21-nucleotide duplex RNA (double-stranded DNA) of dSmurf (15). Cells were induced by 0.5 mM CuSO4 for 24 h and then harvested for Western blot analysis.

For ubiquitination assay, 293T cells were cotransfected by His-tagged Myc-dSmurf and Mad 293T cells were cotransfected by His tagged MAD, HA-tagged ubiquitin, and c-Myc-tagged dSmurf. Proteasome inhibitor MG-132 was added to the transfected cells to prevent
conjugated fluorescent secondary antibodies as described previously and rabbit anti-pMAD (1:250) with fluorescein isothiocyanate- and Cy3-

For targeted expression, flies were generated by P-element-mediated germline transformation (16). pUAST-FLAG and dSmurf(C1029A) were cloned in

nated MAD as described previously (9).

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RESULTS

WW2/WW3 Domains of dSmurf Mediates Its Specific Interaction with MAD—By homology probing, we identified dSmurf cDNA that encodes a polypeptide of 1061 amino acid. dSmurf belongs to the HECT-class ubiquitin E3 ligase family (Fig. 1A) and displays a close homology to human Smurf2 (9–11) and Smurfl (8). During the course of this study, Podos et al. (1) also identified dSmurf in a genetic screen (1). As a first step to determine whether dSmurf modulates DPP signaling in Drosophila, we investigated the physical interaction of this puta-

tive ubiquitin E3 ligase with members of the Smad family in Drosophila. We found that dSmurf only interacted with MAD, but not with dSmad2, Medea, or Dad. (Fig. 1B). Therefore, unlike its mammalian homologs Smurf1/2 that bind to both R-Smads and I-Smads, dSmurf specifically interacts with DPP-specific MAD.

To locate the interacting domains of dSmurf, we generated deletion and point mutations in dSmurf and assessed their interaction of MAD. The result showed that the entire WW2/WW3 region of dSmurf (amino acids 513–612) was required for the interaction with MAD, whereas the WW2 or WW3 domain alone did not interact with MAD (Fig. 1C). Notably, the same WW2/WW3 region with point mutations in WW2 domain (W541F/P544A) completely lost the interaction with MAD. These data suggest that the interaction of dSmurf binds to MAD through the WW2/WW3 region of dSmurf (amino acids 513–612).

DPP Receptor Activation Augments dSmurf-MAD Interaction—We next assessed the MAD-dSmurf interaction in vivo. To prevent MAD degradation, we used the C1029A mutant of dSmurf to determine its interaction with Smads. The C1029A mutant carries a cysteine-to-alanine substitution at amino acid
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FIG. 3. dSmurf ubiquitates MAD. 293T cells were transfected with expression plasmids for His-tagged ubiquitin, and Myc-dSmurf. His-MAD was pulled down from cell lysates using nickel-NTA-agarose beads (Ni-NTA ppt) and then subjected to SDS-PAGE and Western blotting analysis using anti-HA antibodies to detect the pool of ubiquitinated MAD (upper). Levels of MAD in cells were subjected to SDS-PAGE and Western blotting analysis using anti-MAD and anti-Myc antibodies. (bottom), dSmurf(CA) is the catalytically inactive mutant of dSmurf with C1029A point mutation. Ubiquitinated MAD is marked.

Fig. 4. dSmurf degrades MAD proteins in cells. A, dSmurf induces proteasome-dependent degradation of MAD. Drosophila S2 cells were transfected with indicated MAD, with or without Myc-tagged dSmurf and treated with MG132 as indicated. Cell lysates were subjected to SDS-PAGE and Western blotting analysis using anti-MAD and anti-Myc antibodies. Note that caTKV further promoted dSmurf-induced MAD degradation (lane 4). B, MAD degradation requires the HECT catalytic activity of dSmurf. 293T cells were transfected with expression plasmids for FLAG-tagged MAD and increasing doses of Myc-dSmurf (wild type or C1029A mutant). Cell lysates from transfected cells were subjected to anti-FLAG Western blot to detect MAD and anti-Myc immunostaining to detect dSmurf. C, depletion of dSmurf expression stabilizes MAD. Drosophila S2 cells were transfected with Myc-tagged dSmurf and MAD and treated with increasing doses (20, 40, and 100 nM) of siRNA against dSmurf as indicated. Cell lysates were subjected to SDS-PAGE and Western blotting analysis using anti-MAD and anti-Myc antibodies. D, depletion of dSmurf expression stabilizes pMAD. Drosophila S2 cells were transfected with caTKV and treated with 100 nM si-dSmurf. Level of phospho-MAD was analyzed by Western blotting using anti-P-Smad1 antibody.

residue 1029 that is essential for catalysis. As shown in Fig. 2A, dSmurf coimmunoprecipitated with MAD, but not with dSmad2, Medea, and Dad, in agreement with the interaction pattern from yeast two-hybrid analysis. caTKV, constitutively active form of type I receptor Thick-veins (18, 19), enhanced the interaction between MAD and dSmurf (Fig. 2, A and B).

To examine the importance of the phosphorylation motif SSXS, we generated double mutations in MAD at Ser-433 and Ser-435, which were changed into Ala and presumably unphosphorylatable by TKV. Result in Fig. 2B clearly showed that the interaction between this mutant, named MAD(SA), and dSmurf was not detectable regardless of the presence of caTKV, whereas wild type MAD strongly interacted with dSmurf in the presence of caTKV. Furthermore, dSmurf also had stronger binding to endogenous phospho-MAD, which can be further enhanced by proteasome inhibitor MG-132 (Fig. 2C). Interestingly, dSmurf also interacts with dSmad2 in the presence of caActR1B (Fig. 2D), suggesting that dSmad2 phosphorylation is necessary for its binding to dSmurf (despite the absence of such interaction in yeast two-hybrid assay as shown in Fig. 1). Therefore, data presented in Fig. 2 clearly demonstrate the important involvement of SSXS phosphorylation in Smad interaction with dSmurf.

dSmurf Mediates Ubiquitination of MAD—To dissect the function of dSmurf as a ubiquitin E3 ligase, we tested whether dSmurf stimulated the ubiquitination of MAD. As shown in Fig. 3, wild type dSmurf was capable of enhancing ubiquitination of MAD protein (lane 3), while no ubiquitination was observed with MAD alone (lane 1). Markedly, the C1029A mutant of dSmurf could not conjugate HA-ubiquitin to MAD (lane 4). This suggests that the catalytic activity of dSmurf is required for the ubiquitination of MAD.

dSmurf Promotes Proteolytic Degradation of MAD—Having established that dSmurf mediated MAD ubiquitination, we next examined the MAD degradation in Drosophila S2 and human 293T cells. In S2 cells, the MAD protein level was reduced dramatically by overexpressing dSmurf (Fig. 4A). Similarly, dSmurf degraded MAD in 293T cells in a dose-dependent manner (Fig. 4B). In sharp contrast, the C1029A mutant did not decrease the steady state level of MAD (Fig. 4B).

We next examined the role of phosphorylation of MAD in dSmurf-mediated degradation. It was clear that activated type I receptor further enhanced the degradation of MAD protein in dSmurf-overexpressing cells (Fig. 4A, compare lane 4 with lane 3), consistent with increased phosphorylation level (data not shown). Most notably, addition of proteasome inhibitor MG-132 inhibited MAD degradation caused by dSmurf (Fig. 4A, lane 5). We then took a loss-of-function approach to examine the MAD degradation by depleting the dSmurf expression. S2 cells were transfected with MAD and Myc-dSmurf and treated with

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or without a 21-nucleotide double-stranded RNA that targets dSmurf (si-dSmurf). We could detect more MAD protein with increased concentration (20, 40, and 100 nM) of the siRNA (Fig. 4C). Simultaneously, reduced dSmurf protein level was detected by anti-Myc antibody (lower panel, Fig. 4C). Importantly, an increased endogenous phospho-MAD protein level was detected after 100 nM si-dSmurf treatment (Fig. 4D), suggesting the silencing of endogenous dSmurf by si-dSmurf.

Overexpression of dSmurf Leads to Loss of Phospho-MAD and Disruption of Imaginal Disc Development—DPP signaling is essential for patterning of imaginal discs. DPP is expressed along the anterior-posterior border of the wing disc and activated phospho-MAD is expressed in wider area around the AP border. Using UAS-Gal4 targeted expression system (20), we tested whether overexpression of dSmurf along the AP border can reduce or abolish phospho-MAD. As expected, when dSmurf expression was targeted to the AP border using ptc-Gal4, there was a strong reduction of phospho-MAD level in the ptc expression domain (Fig. 5). ptc-Gal4;UAS-dSmurf (ptc>dSmurf) flies failed to eclose due to severe developmental defects. Phaorate adult flies removed from the pupal case showed rudimentary wings (Fig. 5L) and truncated legs (not shown), indicating strong reduction of DPP signaling. In contrast, overexpression of catalytically inactive dSmurf in ptc>dSmurf(C1029A) showed no significant defects in appendages. Wing discs from ptc>dSmurf(C1029A) showed slight increases in the level of phospho-MAD (Fig. 5, E–G), suggesting that dSmurfC1029A may titrate the function of normal dSmurf.

DISCUSSION

In this study, we have elucidated the mechanism underlying dSmurf-MAD interaction and characterized the biological function of dSmurf in Drosophila. dSmurf preferentially interacts with phosphorylated MAD (pMAD). The preferential binding of dSmurf to pMAD is similar to a previously reported Smurf2 binding to phosphorylated Smad2 (9). Furthermore, for the first time we demonstrate that mutation in the SSXS motif of MAD abolishes its binding to dSmurf, highlighting the important role of MAD phosphorylation in its degradation. This phosphorylation-dependent MAD–dSmurf interaction is contrary to a previously proposed model that Smurfs target nonphosphorylated Smad1/4 for degradation and thus reduces the cell competence to BMP/DPP signals (1, 8). The phosphorylation-dependent mechanism, as also discussed (21), is consistent to the disappearance of pMAD in dSmurf-overexpressing transgenic flies (Fig. 5) and also agrees with the increased pMAD level in dSmurf-null mutants (1). While at present it is not clear how the phosphorylation controls the dSmurf-mediated MAD degradation, three possible mechanisms can be envisioned. First, phosphorylation of the SSXS motif disrupts the intrinsic MH1-MH2 interaction and results in an exposed PPXY motif in the linker region for dSmurf binding. Second, dSmurf interaction with MAD depends on the nuclear entry of MAD, which is induced by ligand. The third possibility is that the phosphorylated SSXS motif may be directly involved in MAD binding to dSmurf. Besides PPXY motif, phosphoserine (Ser(P)) residues also serve as ligand for WW domain (22). It will be interesting to determine whether one of WW domains of dSmurf can directly bind to the phosphorylated SSXS motif of MAD.

dSmurf also interacts with dSmad2 dependent of receptor activation. The binding specificity of dSmurf is similar to that of its structurally related Smurf2 which binds both BMP- and activin-activated R-Smads in vertebrates (9, 10). One remaining question is whether dSmurf also targets dSmad2 for degradation. Since activin signaling through dSmad2 appears to have no effects on patterning of wing development (23), the hypothetical dSmurf-mediated dSmad2 degradation in the wing imaginal disc should have no contributions to the severe phenotype observed in the dSmurf transgenic flies. It is the disappearance of phospho-MAD that completely shuts down DPP signaling during wing development.

Unlike its vertebrate homologs, dSmurf fails to interact with DAD, the inhibitory Smad in fly. In vertebrates, Smurfs target nonphosphorylated R-Smads for degradation. Since activin signaling through dSmad2 appears to have no effects on patterning of wing development (23), the hypothetical dSmurf-mediated dSmad2 degradation in the wing imaginal disc should have no contributions to the severe phenotype observed in the dSmurf transgenic flies. It is the disappearance of phospho-MAD that completely shuts down DPP signaling during wing development.

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REFERENCES

1. Podos, S., Hanson, K., Wang, Y., and Ferguson, E. (2001) Dev. Cell 1, 567–578
2. Raferty, L., and Sutherland, D. (1999) Dev. Biol. 210, 251–268
3. Roberts, A. B., and Derynck, R. (2001) Science’s STKE http://stke.sciencesmag.org/cgi/content/full/stke.2001.113.PE43
4. Padgett, R., and Patterson, G. (2001) Dev. Cell 1, 343–349
5. Sekelsky, J., Newfeld, S., Raferty, L., Chartoff, E., and Gelbart, W. (1995) Genetics 139, 1347–1358
6. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
7. Ciechanover, A., Orien, A., and Schwartz, A. L. (2000) Bioessays 22, 442–451
8. Zhu, H., Kavask, P., Abdollah, S., Wrana, J. L., and Thomsen, G. (1999) Nature 400, 687–693
9. Lin, X., Liang, M., and Feng, X.-H. (2000) J. Biol. Chem. 275, 36818–36822
10. Zhang, Y., Chang, C., Gehling, D., Hemmati-Brivanlou, A., and Derynck, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 974–979
11. Kavask, P., Rasmussen, R. K., Causing, C., Bonni, S., Zhu, H., Thomsen, G., and Wrana, J. (2000) Mol. Cell 6, 1365–1375
12. Elissew, T., Fukusho, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001) J. Biol. Chem. 276, 12477–12480
13. Feng, X.-H., Lin, X., and Derynck, R. (2000) EMBO J. 19, 5178–5193
14. Feng, X.-H., Liang, Y.-Y., Liang, Z., Zhao, W., and Lin, X. (2002) Mol. Cell 9, 133–143
15. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschi, T. (2001) Nature 411, 494–498
16. Spradling, A., and Rubin, G. (1992) Science 258, 341–347
17. Cho, K. O., and Choi, K. W. (1998) Nature 396, 272–276
18. Brummel, T., Twombly, V., Marques, G., Wrana, J., Newfeld, S., Attisano, L., Massague, J., O’Connor, M., and Gelbart, W. (1994) Cell 78, 255–267
19. Nellen, D., Affolter, M., and Basler, K. (1994) Cell 78, 255–267
20. Brand, A., and Perrimon, N. (1993) Development (Camb.) 118, 401–415
21. Arora, K., and Warrior, R. (2001) Dev. Cell 1, 441–442
22. Lu, P., Zhou, X., Shen, M., and Lu, R. (1999) Science 283, 1325–1328
23. Brummel, T., Abdollah, S., Haerry, T., Shimell, M., Merriam, J., Raferty, L., Wrana, J., O’Connor, M. (1999) Genes Dev. 13, 98–111
