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SP-RING for SUMO: New Functions Bloom for a Ubiquitin-like Protein

Minireview

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SUMO is covalently linked to a variety of cellular proteins. Three groups now describe related E3-like factors that enhance transfer of SUMO to specific proteins. This family of factors includes proteins important for chromosome condensation, signal transduction, and ion channel biogenesis.

Covalent attachment of one protein to another represents one of the more prominent posttranslational modifications—in terms of both size and ubiquity—to which eukaryotic proteins are subject. Ubiquitin is the most familiar of the proteinaceous protein modifiers, and the enzymology of its activation and transfer to target molecules has been studied extensively for over two decades. Much more recently, a sizeable group of ubiquitin-related proteins have come to light, often as a result of genome sequencing efforts. Many of these proteins consist of ubiquitin-related domains that are stably built into larger structures, and in some cases it is likely these domains derive from independent protein lineages that have converged on the ubiquitin fold because of its intrinsic structural utility. However, at least a dozen distinct ubiquitin-like proteins (Ubls) do precisely what ubiquitin is so well known for doing: they form covalent attachments to other macromolecules.

SUMO, a Ubl with Many Targets

One of the most intriguing of the Ubls is SUMO (small *u*biquitin-related *mo*difier) (Melchior, 2000). In vertebrates there are several variants of SUMO (SUMO-1, -2, and -3), while in the budding yeast *Saccharomyces cerevisiae*, there is only one, encoded by the *SMT3* gene, which is essential for progression through the cell cycle. The only known substrates to date for SUMO in yeast are several members of a family of GTP binding proteins called septins, which assemble at the neck between mother and daughter cells and are essential for cell separation (Takahashi et al., 1999; Johnson and Blobel, 1999). The septins are specifically sumoylated during the G_2/M phase of the cell cycle, which is precisely the point where cells arrest if they are unable to conjugate SUMO. Surprisingly, however, preventing septin sumoylation by mutating all the major SUMO addition sites does not block, or even delay, the cell cycle (Johnson and Blobel, 1999).

The first substrate found for SUMO was the vertebrate nucleocytoplasmic transport factor RanGAP1, and a number of additional vertebrate substrates were identified soon thereafter (Melchior, 2000). But it was in yeast that the SUMO-activating and -conjugating enzymes, as well as the SUMO-deconjugating enzymes, were first described (Figure 1; reviewed in Melchior, 2000). As with ubiquitin, the C-terminal carboxyl group of SUMO appears to be activated by adenylation, in this case by the heterodimeric "E1-like" enzyme Uba2-Aos1; the SUMO-AMP is thought to remain tightly bound to Uba2-Aos1. In a second step, a thioester is formed between Uba2 and SUMO. The activated SUMO is transferred—again like ubiquitin—to a cysteine side chain of the "E2-like" protein called Ubc9. Ubc9 then transfers its thioester-linked SUMO to a substrate lysine residue. Both Uba2 and Aos1 contain extended regions of sequence similarity to the E1 for ubiquitin, and Ubc9 is so similar to ubiquitin-conjugating enzymes that it was originally mistaken for one.

To those who cut their teeth studying ubiquitin, two features of SUMO ligation have seemed very peculiar. The first is the existence of a recognizable consensus sequence—ΨKxE, where Ψ is an aliphatic residue—surrounding the substrate lysine(s) that is sumoylated. No such consensus sequence has ever been found for ubiquitination targets, and for many substrates, the ubiquitin conjugation enzymes show a remarkable indifference toward the particular lysine that is to be modified. The second unusual aspect of sumoylation is that the E1 and E2 enzymes (together with ATP and SUMO) appear to be sufficient in vitro for relatively robust modification of proteins at precisely the lysines that are preferred in vivo. For ubiquitin, an additional factor, called an E3 or ubiquitin-protein ligase, is almost always necessary for efficient substrate ubiquitination, both in vitro and in vivo. An E3 has been defined as "an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, from a thiolester intermediate to amide linkages with proteins or polyubiquitin chains." (Hershko and Ciechanover, 1998). These differences have raised the question of whether SUMO ligation to proteins is mechanistically distinct from ubiquitin ligation. In particular, it seemed possible that no E3-like factors were needed for substrate-specific sumoylation.

New findings reported by Johnson and Gupta in the past issue of *Cell* (Johnson and Gupta, 2001), by Yasuda and colleagues in *Molecular Cell* (Kahyo et al., 2001), and by Takahashi et al. in *Gene* (Takahashi et al., 2001) now demonstrate that the apparent dispensibility of E3-like factors for protein sumoylation was illusory. All three groups identified structurally related factors from either yeast or human cells that are capable of greatly stimulating substrate-specific SUMO ligation both in vivo and in vitro. Their results further highlight the mechanistic parallels between protein sumoylation and ubiquitination, but they also raise new questions about how SUMO is ligated to particular proteins and what functions sumoylation serves.

E3-like Proteins for SUMO Conjugation

Kahyo et al. and Takahashi et al. both found their way to their E3-like factors via yeast two-hybrid interaction

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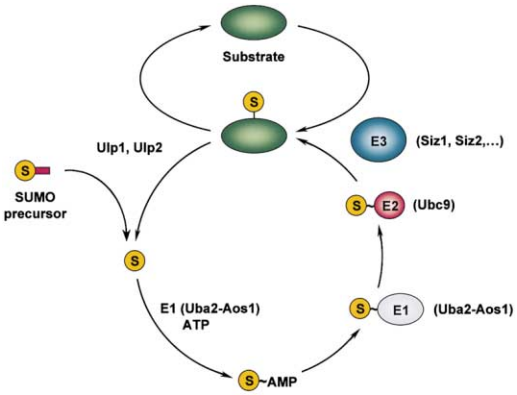

Figure 1. The SUMO Cycle

Enzyme names are from *S. cerevisiae*, where most of the components were first described.

screens for proteins that bound to SUMO. Johnson and Gupta, on the other hand, simply made an educated guess based on an obscure entry logged in the *Saccharomyces* Genome Database website, which noted an otherwise uncharacterized protein, Nfi1, that was said to interact with the septin Cdc12. Although deletion of the *NFI1* gene (subsequently renamed *SIZ2*) had no impact on the level of sumoylated septins, removal instead of a related gene, *SIZ1/ULL1*, severely reduced SUMO attachment to all three of the septins that are normally sumoylated (Johnson and Gupta, 2001). Takahashi et al. (2001) arrived at *SIZ1/ULL1* by a similar route following the observation of a two-hybrid interaction between Nfi1/Siz2 and SUMO. The mammalian proteins to which Siz1 and Siz2 are most closely related are the PIAS (protein inhibitor of activated STAT) proteins, and it was PIAS1 that Kahyo et al. identified as a potential E3-like protein for sumoylation of the p53 tumor suppressor in human cells.

What led all these investigators to the idea that Siz1, Siz2, and PIAS1 might be E3-like factors was the modest similarity of a conserved internal segment of these proteins to the RING finger, a motif that defines the largest subfamily of ubiquitin E3s (Hershko and Ciechanover, 1998). RING fingers bind a pair of zinc atoms in a distinctive "cross-brace" arrangement of coordinating Cys and His residues, creating a globular domain that can directly bind E2s. However, the Siz/PIAS RING (SP-RING) lacks two of the cysteines that are conserved in all known RING E3s, so it remains to be demonstrated that it has the same general structure as a classical RING. It is noteworthy that mutation of several zinc-coordinating cysteines in the BRCA1 RING finger does not abolish zinc binding (Brzovic et al., 2001), and that the RING in Mdm2, a ubiquitin E3, has a threonine instead of a cysteine at one of the coordinating positions (see Kahyo et al., 2001). Furthermore, a motif called the U box is predicted to have a fold very similar to that of a RING finger despite lacking all the metal-coordinating residues; recently, several U box proteins were shown to have E3 ubiquitin-ligase activity (Hatakeyama et al., 2001). It will be interesting to determine the three-dimensional structure of the SP-RING to see if it coordinates one or two (or any) zinc ions and to verify that it folds into a RING-like conformation.

In the most complete of the three new studies, Johnson and Gupta (2001) provide convincing evidence that Siz1 is an E3-like protein that promotes SUMO attachment to specific proteins. Recombinant E1, E2, and Siz1 purified from *E. coli* are able to synthesize polySUMO chains from monomeric SUMO; more importantly, since the significance of SUMO chains is unknown, the same set of proteins can catalyze sumoylation of a highly purified septin preparation from yeast. Siz1 binds to the E2 Ubc9 and appears to bind, directly or indirectly, to septins (Takahashi et al., 2001). In vivo, Siz1 is specifically required for septin modification whereas Siz2 is needed for the sumoylation of an unidentified ~27 kDa protein. Thus, all the criteria defining an E3 are satisfied. From the other two studies one can add the conclusions that the SP-RING domain is necessary for stimulation of SUMO ligation, and in particular, is required for Ubc9 binding, which is reminiscent of ubiquitin E3 RING finger association with E2. Collectively, these results strongly imply that SP-RINGs function in sumoylation in a manner that is mechanistically similar to their ubiquitin pathway cousins.

A clear difference remains, however. At high concentrations of E1 and E2, the Siz1 protein is no longer required for efficient substrate modification in vitro, and even at lower E1 and E2 levels, a small amount of septin sumoylation was still detectable in the absence of Siz1 in vitro and in vivo (see Takahashi et al., 2001). In contrast, ubiquitin E3s are essential for substrate targeting. Does this mean that the ubiquitin and SUMO E3s function in fundamentally different ways? The answer is—probably not. Ubiquitin RING E3s are thought to act by bringing the E2 and substrate into close spatial proximity to allow ubiquitin transfer from the E2 to the attacking lysine side chain of the substrate, so a contribution of the E2 to substrate binding is not unreasonable. It is likely that a continuum exists in the distribution of substrate binding interactions deriving from the E2 versus E3 components in E2/E3 ubiquitin-conjugation complexes. The SUMO pathway may be shifted toward the end of the spectrum where a substantial part of the binding energy comes from the E2. Having the E2 play a larger role in substrate recognition might be the more "primitive" situation among the Ubl ligation systems since in the ancestral modification system(s), E2-like proteins probably targeted substrates without an E3. E3-like factors likely arose later, allowing for enhanced or expanded specificities—or in some cases taking over the task of substrate discrimination almost entirely (Hochstrasser, 2000). Analysis of additional SUMO substrates and other Ubl conjugation systems will be necessary to test these conjectures.

What Does SUMO Modification Do*?*

Although there are many similarities in the biochemistry of ubiquitin and SUMO ligation, we are at an early stage in deciphering what SUMO attachment actually does for a protein. Most generally, we would like to know the molecular basis for the functional differences between SUMO-linked proteins and their nonconjugated forms. In addition, however, it will be important to determine exactly which proteins are sumoylated, when and where the modification occurs, and how the sumoylation of

individual substrates contributes to a particular physiological process, such as cell cycle progression or nucleocytoplasmic trafficking.

Concerning mechanisms for the functional alteration of proteins by SUMO, two general models have gained currency. The first and most obvious idea is that a SUMO-protein conjugate can acquire an altered affinity for a particular ligand. For example, sumoylated RanGAP1 appears to bind preferentially to the nuclear pore complex (NPC) relative to either free SUMO or RanGAP1 (Melchior, 2000). The enhanced binding could be due to increased avidity for the NPC provided by multiple binding sites in both SUMO and RanGAP1, interference by SUMO attachment with a factor that limits RanGAP1 access to the NPC, conformational changes in either partner protein that expose or create an NPC binding site, or some combination of these mechanisms. The second model for SUMO function, for which there is also experimental evidence, holds that SUMO ligation to a substrate can block attachment of ubiquitin (or another Ubl) to the same substrate, possibly by competition for the same lysine residue. Support for such a mechanism comes from studies of the IκBα and Mdm2 proteins. A large fraction of Mdm2, for instance, is normally sumoylated on a specific lysine in the Mdm2 RING finger (Buschmann et al., 2000). In its nonsumoylated form, the Mdm2 ubiquitin ligase is prone to catalyze its own polyubiquitination and destruction. Because Mdm2 negatively regulates the p53 tumor suppressor, such a reduction in Mdm2 sumoylation will tend to increase levels of p53 and p53's growth inhibitory activity. Indeed, Ronai and colleagues found that inflicting DNA damage on cells by exposure to radiation, which is known to enhance p53 stability, impairs Mdm2 sumoylation (or enhances its desumoylation) and this in turn correlates with an increase in p53 levels (Buschmann et al., 2000).

Unfortunately, for situations where SUMO is not simply competing with ubiquitin or another Ubl for modification of a protein, the physiological significance of protein sumoylation has generally remained obscure. RanGAP1 again serves as an instructive example. Although sumoylated RanGAP1 clearly concentrates at the cytoplasmic fibrils of the NPC and such localization depends on its sumoylation, why RanGAP1 must localize to the NPC and whether a failure to do so has any functional consequence are still not known with certainty (Melchior, 2000). Yeast RanGAP1, for comparison, is neither sumoylated nor localized to the NPC. Other substrates are even more problematic. The p53 transcription factor, which was the substrate used by Kahyo et al. to evaluate PIAS1 sumoylation-enhancing activity, is subject to a low level of sumoylation in vivo, and the major modification site has been mapped. In the original reports on p53 sumoylation, mutation of the target lysine to an arginine, which blocks SUMO addition, prevented the modestly enhanced transactivation activity of p53 that is caused by augmenting cellular SUMO levels, but several laboratories have not been able to reproduce this result (Kwek et al., 2001 and references therein). Even the elegant studies on yeast septin sumoylation have failed to reveal the purpose of this modification (Johnson and Blobel, 1999). Clearly, understanding SUMO function at a molecular level will require considerable additional analysis.

In Vivo Function of SUMO E3s

Similarly, very little is known about the physiological roles of Siz1 and Siz2. Neither Siz protein is essential for yeast viability, in contrast to several other enzymes in the yeast SUMO pathway, although the *siz1*Δ *siz2*Δ double mutant grows poorly at low temperatures. Interestingly, Siz1 was initially identified in studies that linked it genetically to the condensin complex, which orchestrates mitotic chromosome condensation (Strunnikov et al., 2001). Consistent with this, most of Siz1 localizes to the nucleus. In contrast to the relative paucity of information on yeast SP-RING protein biology, a string of papers has detailed possible biological functions for the various metazoan versions (Table 1). The closest link to the yeast work on Siz1 is a recent report that the lone member of the PIAS family in *Drosophila*, called dPIAS, Su(var)2-10, or Zimp, is required for normal mitotic chromosome condensation and interphase chromosome organization (Hari et al., 2001). Loss of dPIAS is lethal, and it has been shown to be required for normal blood cell and eye development (Betz et al., 2001).

In mammals, the PIAS proteins, which are encoded by a small gene family, were first identified as transcriptional coregulators (Greenhalgh and Hilton, 2001). In the JAK-STAT signal transduction pathway, assembly of specific receptor complexes after ligand binding leads to activation of receptor-associated kinases of the Janus (JAK) family, which then recruit and phosphorylate DNA binding transcription factors of the STAT family. This results in dimerization of the STATs followed by their translocation into the nucleus and transcriptional activation. Downregulation of the pathway occurs by several means, including direct binding of the activated STAT dimer by members of the PIAS family, which in most—but not all—cases prevents STAT-DNA binding. PIAS proteins can also play positive roles in transcription, and one of the PIAS proteins, PIAS1 or GBP, has been isolated by its ability to bind to a nucleolar RNA helicase, called Gu/RNA helicase II (Valdez et al., 1997). Remarkably, when GBP associates with the helicase, proteolytic cleavage of the helicase is triggered. As in all of the other examples above, the participation of SUMO ligation in these biochemical events is not yet known.

Most tantalizing of the SP-RING factors with respect to the SUMO pathway is a rat protein called ARIP3 (androgen receptor interacting protein), which appears to be the ortholog of PIASx in humans (Moilanen et al., 1999). Unlike the aforementioned SP-RING binding proteins, the protein to which ARIP3 was found to bind, the androgen receptor (AR), has also been shown to be sumoylated on a specific lysine in vivo (Poukka et al., 2000). Mutation of this residue blocks sumoylation and enhances transcriptional activity of AR, suggesting that SUMO modification negatively regulates AR activity. The target lysine is part of a ΨKxE consensus site, which, interestingly, is identical to something called the "transcriptional synergy control motif" (see Poukka et al., 2000). Not only AR but several other members of the steroid receptor superfamily have this motif, raising the possibility that SUMO modification may modulate a host of different nuclear receptor transcription factors, with the SP-RING proteins presumably helping to direct the modification.

Table 1. Regulatory Pathways Linked to the SP-RING Family of (Putative) SUMO Ligases

SP-RING Protein	Alternative Names	Organism	Pathways or Substrates	Ref.
Siz1	Ull1, YDR409w	*S. cerevisiae*	septins (Cdc3, Cdc11, Sep7)	a, b
Siz2	Nfi1, YOR156c	*S. cerevisiae*	unknown; required with Siz1 for growth at low temperature	a, b
PIAS1	GBP	*H. sapiens*, *M. musculus*	binds STAT1, Gu/RNA helicase II; stimulates p53 sumoylation	c, d, e
PIAS3	KChAP	*M. musculus* *H. sapiens* *R. norvegicus*	binds K^+ channels, STAT3	c, f
PIASx	ARIP3 Miz1, Disabled 2-interacting protein	*M. musculus* *H. sapiens* *R. norvegicus*	binds STATx; androgen receptor, which is sumoylated; mouse Disabled-2 p67 isoform; Msx2	c, g
dPIAS	Su(var)2-10, Zimp	*D. melanogaster*	binds *Drosophila* stat92E; required for chromosome condensation, structure; essential for development	h, i

[a]Johnson and Gupta (2001); [b]Takahashi et al. (2001); [c]Greenhalgh and Hilton (2001); [d]Kahyo et al. (2001); [e]Valdez et al. (1997); [f]Kuryshev et al. (2000); [g]Moilanen et al. (1999); [h]Hari et al. (2001); [i]Betz et al. (2001).

Although the SP-RING proteins loiter primarily in the nucleus and seem to have many roles there, yeast Siz1 can also localize near the plasma membrane in the bud neck region during part of the cell cycle. Furthermore, a mammalian SP-RING factor, PIAS3 or KChAP, has been found to interact transiently with a subset of voltage-regulated potassium channels (Kuryshev et al., 2000). KChAP is believed to function as a kind of molecular chaperone, enhancing channel biogenesis by a mechanism that has not yet been ascertained. It will clearly be of interest to determine if the putative SUMO E3 activity of the protein contributes to this unusual chaperoning property.

What Next?

The discovery of SUMO ligation activity for the widespread and highly conserved SP-RING family of proteins is an exciting development for the many researchers who have been working on these proteins in various biological contexts. What is clearly missing from all the studies to date is an understanding of how this enzymatic activity of these proteins relates to their specific biological functions. As the painstaking analysis of yeast septin sumoylation has made clear, establishing such relationships can sometimes be quite frustrating. Other major questions are how many SUMO E3s exist and will they all be part of the same structural family. Yeast appear to have no SP-RING proteins other than Siz1 and Siz2, yet sumoylation is still detected in vivo in the absence of these two enzymes. Although it is possible that this reflects E3-independent SUMO conjugation by Ubc9, the existence of additional, non-SP-RING E3s seems more likely. Several classical RING proteins from yeast, for example, have been isolated by large scale two-hybrid screens with SUMO as bait, and non-RING E3s are also possible. What is clear at this point is that SUMO ligation is emerging as a critical factor in cellular processes ranging from cell cycle regulation to transcriptional control, and we can expect to hear much more about this fascinating protein modification system in the coming years.

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