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Protein SUMOylation modulates calcium influx and glutamate release from presynaptic terminals

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Abstract

Posttranslational modification by small ubiquitin-like modifier (SUMO) proteins is emerging as an important regulatory mechanism for neuronal function and dysfunction. Although multiple potential presynaptic SUMOylation substrate proteins have been proposed from sequence analysis the functional consequences of presynaptic SUMOylation have not been determined. Here we show that SUMOylation of presynaptic proteins modulates neurotransmitter release. Increasing protein SUMOylation by entrapping recombinant SUMO-1 in synaptosomes decreased glutamate release evoked by KCl whereas decreasing SUMOylation with the SUMO-specific protease SENP-1 enhanced KCl-evoked release. In contrast, SUMO increased and SENP-1 decreased synaptosomal glutamate release evoked by kainate stimulation. Consistent with these results, SENP-1 increased Ca^{2+} influx into synaptosomes evoked by KCl whereas it decreased kainate-induced Ca^{2+} influx. These results demonstrate that, in addition to postsynaptic effects, protein SUMOylation acts to modulate neurotransmitter release and thereby regulate synaptic function.

Keywords

neurotransmitter release; SENP-1; SUMO; synaptic transmission; synaptosome

Introduction

Small ubiquitin-like modifier (SUMO) proteins are ~11 kD proteins that are covalently attached to lysine residues on target proteins, modulating the functional properties of the substrate. SUMOylation can modify various aspects of protein function in a substrate-specific manner (Geiss-Friedlander & Melchior, 2007; Martin *et al.*, 2007b) and is essential for eukaryotic cell viability (Hayashi *et al.*, 2002; Nacerddine *et al.*, 2005). Multiple SUMOylated proteins are present at synapses and SUMOylation of the kainate receptor subunit GluR6 at postsynaptic sites plays a key role in agonist-dependent endocytosis (Martin *et al.*, 2007a). Furthermore, there is increasing evidence that SUMOylation plays important roles in several neuropathologies (Dorval & Fraser, 2007; Martin *et al.*, 2007b) and that SUMOylation may be a neuroprotective mechanism in ischaemia (Lee *et al.*, 2007; Cimarosti *et al.*, 2008; Yang *et al.*, 2008).

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Mammals possess three active SUMO isoforms (SUMO-1 to -3) that are initially synthesised as inactive precursors that must be cleaved by the activity of a family of SUMO-specific cysteine proteases (SENPs) before conjugation to substrate proteins can occur (Geiss-Friedlander & Melchior, 2007; Martin *et al.*, 2007b; Mukhopadhyay & Dasso, 2007). This cleavage step exposes a diglycine (GG) motif at the C-terminus of the SUMO protein, which can then be conjugated via an enzymatic pathway analogous to that of the ubiquitin pathway. Mature SUMO proteins are first activated for conjugation in an ATP-dependent manner by a heterodimer of SAE1/SAE2 before they are passed to the sole SUMO-conjugating enzyme, Ubc9. Ubc9, often in conjunction with one of multiple SUMO-ligating enzymes, then catalyses conjugation of SUMO to the substrate (Martin *et al.*, 2007b; Geiss-Friedlander & Melchior, 2007). SUMOylation is highly reversible and the SENP enzymes responsible for SUMO maturation also cleave SUMO from substrate proteins (Mukhopadhyay & Dasso, 2007).

A wide range of pathways in the presynaptic nerve terminal orchestrate the amount and duration of vesicular glutamate release and these processes, in turn, underlie activity-dependent changes in synaptic function and plasticity (Stevens, 2004). In addition, dysfunctions of glutamate neurotransmission are important factors in many neurological and psychiatric disorders (Konradi & Heckers, 2003; Spedding *et al.*, 2003).

Synaptosomal preparations are an established and robust model for the study of presynaptic neurotransmitter release (Nicholls & Coffey, 1994). One particularly useful property of synaptosomes is that, during their preparation as the synaptic terminals are sheared from the axons, the internal compartment of the presynaptic bouton is transiently accessible to the external media before it rapidly seals to form a sealed synaptosome. This makes it possible to entrap non-membrane-permeable compounds such as peptides or even large proteins inside synaptosomes to study their effects (Pittaluga *et al.*, 2005).

Here we investigated whether manipulation of the SUMOylation status of presynaptic substrate proteins by entrapping SUMO-1 or the specific SUMO protease SENP-1 influences glutamate release from synaptosomes. Promoting synaptic protein SUMOylation with SUMO-1 reduced Ca^{2+} influx and decreased glutamate release evoked by KCl whereas decreasing SUMOylation with SENP-1 increased Ca^{2+} influx and enhanced KCl-evoked release. Intriguingly, the reverse was observed for kainate-evoked Ca^{2+} influx and glutamate release.

Materials and methods

Plasmids

pET-SUMO-1(1–104) (wild type SUMO-1 precursor), pET-SUMO-1(1–97) (mature conjugatable form), pET-SUMO-1(1–95) (Δ GG-conjugation-deficient form) and pETHis₆-S-tag-SUMO1-GST have been described elsewhere (Martin *et al.*, 2007a). pMax(+)-SENP-1 (accession number BC045639; both wild-type and C603S-mutant) were a gift from S. Goldstein. The SENP-1 fragment, containing the entire isopeptidase catalytic domain (R352-L643), was excised as a XhoI and SalI fragment from pMax(+)-SENP-1. pET-SENP-1(WT) and pET-SENP-1(C607S) were then made by insertion of the SENP-1 fragment into the XhoI site of the pET30a(+) vector (Novagen). The enzymatic activities of the purified recombinant proteins were monitored using His₆-SUMO-1-GST (Martin *et al.*, 2007a) as a substrate.

Expression and purification of recombinant proteins

His₆-tagged proteins were produced in BL21(DE3) *Escherichia coli* and purified with HIS-Select™ Cobalt Affinity Gel (Sigma).

Synaptosome preparation

Male and female adult Wistar rats (200–250 g) were humanely killed by decapitation according to UK Home Office schedule 1 procedures. Synaptosomes were prepared from the pooled cortex and hippocampi using percoll–sucrose density-gradient centrifugation as described and extensively characterised previously (Phillips *et al.*, 2001; Feligioni *et al.*, 2006). To entrap purified recombinant SUMO or SENP proteins in synaptosomes, 2 μM protein was added to the homogenisation buffer immediately prior to homogenisation of the brain (Pittaluga *et al.*, 2005). Based on partition of radiolabelled compounds, 4.5–5% of small membrane-impermeant molecules are partitioned into the synaptosomes (Akerman & Heinonen, 1983; Pittaluga *et al.*, 2005), giving a final synaptosomal concentration of SUMO-1 or SENP-1 of $\sim 100 \text{ nM}$. In these entrapment experiments the cysteine-alkylating agent *N*-ethylmaleimide (NEM), which inhibits SENP activity, was not added. After resealing, the synaptosomes were resuspended in 1.2 mL HEPES buffer (in mM: NaCl, 140; KCl, 3; MgSO_4 , 1.2; CaCl_2 , 1.2; NaH_2PO_4 , 1; NaHCO_3 , 3.5; glucose, 10; and HEPES, 5, pH 7.4; unless stated otherwise all chemicals were from Sigma), and then divided into 1-mL aliquots and gently agitated at 37°C until use.

Synaptosome stimulation

Following 20 min mild agitation at 37°C, agonist or vehicle was added to the final concentration indicated and the suspensions were incubated for 6 min. The experiment was terminated by addition of 1 mL of ice-cold HEPES buffer and immediate centrifugation at 16 000 *g* to pellet the synaptosomes. For NMDA stimulation experiments Mg^{2+} was omitted to avoid the functional inactivation of the receptor.

Glutamate release assays

Synaptosomal glutamate release was monitored using a well-established NADPH⁺ fluorescence method (Nicholls *et al.*, 1987; Rodriguez-Moreno & Sihra, 2004; Kilbride *et al.*, 2008). Synaptosomes were equilibrated at 37°C for 40 min, pelleted and resuspended in fresh HEPES buffer at 37°C in a fluorimeter cuvette. Fluorescence at 450 nm was recorded after an excitation at 340 nm. At $t = 1 \text{ min}$ NADP⁺ was added (final concentration 1 mM) followed at $t = 3 \text{ min}$ by addition of glutamate dehydrogenase (E.C. 1.4.1.3; Sigma; 50 units). The appearance of NADPH fluorescence was then monitored to obtain a baseline. At $t = 8 \text{ min}$ KCl (30 mM), AMPA (50 μM), NMDA (50 μM) or kainate (10 μM) were applied and changes in fluorescence monitored for a further 3 min. All agonists were obtained from Ascent Scientific UK. At the end of each experiment 5 nmol glutamate was added to the cuvette to calibrate the system using the formula:

$$\text{Glutamate (nmol/mg protein)} = (F_{\text{sample}} / F_{\text{standard}}) \times 5 \text{ nmol/mg protein}$$

Ca²⁺-influxfluorimetric assay

Synaptosomes were incubated with gentle agitation in PSS (in mM: NaCl, 145; KCl, 2.6; KH_2PO_4 , 2.4; MgCl_2 , 1.2; glucose, 10; and HEPES, 10; pH was adjusted to 7.4 with NaOH) containing 20 μM of CaCl_2 and 5 μM Fura-2AM for 40 min at 37°C. Control synaptosomal suspensions containing 0.5% DMSO, but no Fura-2AM, were prepared in parallel and used to measure autofluorescence. Synaptosomes were washed at 3000 *g* for 2 min to remove extrasynaptosomal Fura-2AM, resuspended in ice-cold PSS, divided into 200- μL aliquots (each containing 200 μg protein) and stored on ice until use.

Measurements were made with 200 μL of synaptosomes diluted in 1.8 mL of PSS at 37°C using a dual wavelength spectrofluorometer by alternating the excitation wavelengths of 340

and 380 nm. Fura-2 fluorescence at 510 nm was recorded for at least 1 min before addition of agonist applied for 3 min. Signals were calibrated at the end of each experiment by adding 0.1% Triton X-100 in the presence of 1.2 mM CaCl_2 (F_{max}) followed by 7.5 mM EGTA adjusted to pH 8 with 3 mM Tris (F_{min}). Extrasynaptosomal Fura-2AM was determined for each synaptosomal preparation by adding 40 μM MnCl_2 to quench the extracellular fluorescence; Mn^{2+} -quenched fluorescence comprised 7–10% of the total fluorescence at the two wavelengths and was stable for the duration of the experiments. After correcting for the extracellular dye, $[\text{Ca}^{2+}]_i$ was calculated as described previously using a K_d of 224 nM for the Ca^{2+} /Fura-2 complex (Grynkiewicz *et al.*, 1985).

Ultrastructural fractionation

Ultrasynaptic fractions were prepared exactly as described (Feligioni *et al.*, 2006).

SDS-PAGE and Western blots

Samples containing 12 μg of protein were resolved by 10% denaturing SDS-PAGE. Proteins were transferred to PVDF membrane using a semi-dry blotting system and nonspecific binding sites were blocked with Tris-buffered saline–Tween (t-TBS; Tris, 0.02 M; NaCl, 0.137 M; and Tween 20, 0.1%) containing 5% non-fat dried milk. The PVDF membranes were then probed with primary antibodies for 90 min at room temperature. The primary antibodies and dilutions used were: rabbit anti-SUMO-1, 1 : 200 (SantaCruz); and mouse anti-Ubc9, 1 : 200 (BD Transduction Laboratories). Membranes were then washed three times with t-TBS and incubated for 2 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibody : antigoat, 1 : 5000 (Sigma), antimouse, 1 : 5000 (Amersham Bioscience) or antirabbit, 1 : 10 000 (Amersham Bioscience). After a further three washes with t-TBS, immunoreactive bands were detected by enhanced chemiluminescence (Roche). The immunoreactive bands were visualized by exposure to Hyperfilm MP (Amersham Biosciences). Western blots were quantified by densitometric analysis using NIH IMAGE J software.

Results

SUMOylated proteins were present in synaptosomes

We have reported previously that there are multiple SUMOylated proteins in brain (Martin *et al.*, 2007a). Here we extend those findings by reporting that multiple SUMOylated proteins are also present in enriched synaptosomes (Fig. 1A and B). As expected, as the majority of SUMO substrates are nuclear proteins (Geiss-Friedlander & Melchior, 2007); there are fewer SUMO-immunoreactive bands in the synaptosomal preparation than in whole-cell lysate. Despite being a covalent modification, SUMOylation is highly reversible due to the activity of the SENPs (Mukhopadhyay & Dasso, 2007). Interestingly, however, the presence or absence of the cysteine-alkylating agent NEM to block SENP activity had less effect on the total SUMO signal in the synaptosomal fraction than in brain homogenate (Fig. 1A and B). Further, unlike in hippocampal tissue homogenates (Martin *et al.*, 2007a), we did not detect abundant high molecular weight species in the synaptosomes. These results could suggest (i) that large protein SUMO substrates are absent or in low abundance in nerve terminals; (ii) that synaptosome preparation and percoll purification may result in the loss of some high molecular weight proteins; and/or (iii) that synaptosomal SUMOylated proteins are either more stably modified by SUMO or that during the synaptosome preparation particularly labile proteins were deSUMOylated despite the presence of 20 mM NEM throughout the procedure.

A critical enzyme in the SUMOylation pathway is the specific and unique SUMO-conjugating enzyme Ubc9. We have shown that Ubc9 is in synaptosomes, indicating that the

machinery for SUMOylation is present at the presynaptic terminal (Fig. 1C). We next tested whether the subsynaptic distribution of Ubc9 changed following a 50- μ M, 3-min AMPA application, a treatment known to evoke robust glutamate release (Barnes & Henley, 1994). We chose AMPA because it elicits a strong receptor-mediated glutamate release that does not involve kainate receptors, which are themselves SUMO substrates (Martin *et al.*, 2007a) and could therefore be a trigger to recruit Ubc9 for kainate receptor subunit SUMOylation independent of any effects on proteins in the release pathway. There was a marked increase in Ubc9 association with the presynaptic active zone region ($189.70 \pm 9.84\%$; two-tailed Student's *t*-test with three degrees of freedom, $t = 2.416$, $n = 4$, $P = 0.028$ compared to non-AMPA-treated controls), consistent with recruitment or retention of Ubc9 to the presynaptic active zone from the cytosol of the synaptosome (Fig. 1D and E).

Activity-dependent subsynaptic changes in the levels of SUMOylated proteins

Utilizing a synaptic purification technique (Phillips *et al.*, 2001; Feligioni *et al.*, 2006) we analysed the levels of SUMOylated proteins in the presynaptic (active zone region), postsynaptic (PSD) and also nonsynaptic synaptosomal protein fractions under basal and stimulated conditions. Nonsynaptic synaptosomal protein is defined as all protein present in the synaptosome preparation other than in the active zone and PSD. To determine activity-dependant changes in the levels of protein SUMOylation in these fractions we employed four different stimulation protocols, namely KCl and the specific ionotropic glutamate receptor agonists AMPA, NMDA and kainate. Figure 2 shows representative blots of proteins, in the molecular weight range 20–75 kDa, that were clearly and reproducibly resolved.

KCl and AMPA stimulation significantly increased total levels of SUMOylated protein in the presynaptic fraction. Intriguingly, kainate had the opposite effect and significantly reduced presynaptic SUMOylation. Levels of SUMOylated proteins in the postsynaptic fraction were unaltered by any of the treatments. This is probably due to the loss of postsynaptic SUMOylation enzymes during the synaptosomal preparation as the postsynaptic compartment does not usually reseal in our preparations (Feligioni *et al.*, 2006). Interestingly, AMPAR activation reduced the total levels of protein SUMOylation in the nonsynaptic synaptosomal protein fraction.

Entrapping SUMO-1 and or SENP-1 modulates levels of SUMOylation

To define the effects of protein SUMOylation on the modulation of glutamate release we entrapped purified active SUMO-1(GG) or SENP-1 in synaptosomes and NEM was omitted from the buffers. Inclusion of SUMO-1(GG) resulted in a modest increase in observed SUMO immunoreactivity of bands especially between 37 and 10 kDa. Note also the presence of a free tagged SUMO-1 band at ~25 kDa, indicating that SUMO was being supplied in excess. As expected, inclusion of recombinant active SENP-1 decreased the intensity of SUMO-immunoreactive bands due to deSUMOylation of synaptosomal proteins (Fig. 3A). It should be noted, however, that in many systems only a small fraction of substrate is SUMOylated but that this modification has a seeming disproportionately large biological effect (Hay, 2005).

Presynaptic protein SUMOylation regulated neurotransmitter release

KCl application, a standard stimulation protocol to evoke endogenous glutamate release from synaptosomes (Nicholls *et al.*, 1987), elicited the largest increase in presynaptic protein SUMOylation. We therefore tested the effects on glutamate release of preloading synaptosomes with the deSUMOylating enzyme SENP-1 or an inactive point mutant, SENP-1 C603S. As shown in Fig. 3B and C, synaptosomes loaded with wild-type SENP-1 displayed dramatically enhanced glutamate release evoked by a 30 mM KCl stimulus

compared to control ($254.5 \pm 15.8\%$; one-way ANOVA followed by a Bonferroni's test, $n = 9$, $P = 0.013$). In the converse experiments (Fig. 3D and E), synaptosomes loaded with conjugatable SUMO protein (SUMO-1GG) displayed markedly decreased KCl evoked release ($56.00 \pm 7.3\%$; one-way ANOVA followed by a Bonferroni's test, $n = 7$, $P = 0.025$). The nonconjugatable form of SUMO-1 (SUMO-1ΔGG), which cannot covalently bind substrate proteins, had no effect ($97.3 \pm 2.2\%$; one-way ANOVA followed by a Bonferroni's test, $n = 7$, $P = 0.034$), indicating that the effect seen was due to SUMO conjugation and not some noncovalent role of SUMO. Surprisingly, entrapment of the wild-type SUMO-1 precursor also had no effect ($88.0 \pm 9.1\%$; one-way ANOVA followed by a Bonferroni's test, $n = 7$, $P = 0.014$). We attribute this lack of effect to the fact the wild-type SUMO precursor requires cleavage of the last four C-terminal residues by SENP proteins to expose the double glycine motif required for conjugation. Because high levels of SUMO are loaded into synaptosomes this probably overwhelms the endogenous SENP proteins. This form of SUMO will therefore remain immature, and be conjugation-deficient.

Kainate can either evoke glutamate release via ionotropic receptor actions or inhibit glutamate release via metabotropic receptor-like actions (Rodriguez-Moreno & Lerma, 1998; Huettner, 2003; Lerma, 2003; Rozas *et al.*, 2003). We investigated the effects of preloading synaptosomes with SUMO-1 and SENP-1 on the release elicited by $10 \mu\text{M}$ kainate stimulation. There was a pronounced decrease in kainate-evoked glutamate release from synaptosomes preloaded with SENP-1 ($51 \pm 9\%$ compared to control synaptosomes; one-way ANOVA followed by a Bonferroni's test, $n = 5$, $P = 0.022$) but a marked increase in release ($256 \pm 29\%$; one-way ANOVA followed by a Bonferroni's test, $n = 6$, $P = 0.028$) from synaptosomes preloaded with SUMO-1(GG); (Fig. 3F and I).

Protein SUMOylation modulates Ca^{2+} influx into synaptosomes

Calcium influx is a key trigger for neurotransmitter release. We therefore tested whether SENP-1 or SUMO-1 altered the extent of Ca^{2+} entry. There were no KCl or agonist-evoked changes in intracellular Ca^{2+} in Ca-free medium (data not shown), indicating that Ca^{2+} changes were due to influx rather than mobilisation from intracellular stores. Synaptosomes were loaded with the calcium indicator Fura-2AM together with recombinant SENP-1 or SUMO-1 proteins. As shown in Fig. 4, wild-type SENP-1 increased Ca^{2+} influx evoked by KCl or AMPA and decreased it following a kainate challenge. Conjugatable SUMO-1(GG), on the other hand, reduced Ca^{2+} influx evoked by KCl or AMPA and increased the levels of Ca^{2+} influx elicited by kainate.

Discussion

We have shown previously using immunocytochemistry and confocal imaging that both SUMO and the enzymes required for SUMOylation are present in distal dendrites of cultured hippocampal neurones (Martin *et al.*, 2007a). Their presence at the presynaptic terminals, however, has not been determined. Here we demonstrate that (i) SUMOylation occurs at the presynaptic terminal and (ii) it regulates neurotransmitter release. These data indicate for the first time that SUMOylation can play key roles in the modulation of presynaptic components of synaptic transmission in addition to previously reported effects at the postsynaptic membrane (Martin *et al.*, 2007a). Importantly, this study highlights the role of covalent SUMO conjugation at the presynapse. Specifically, the lack of effect of nonconjugatable SUMO and the robust actions of wild-type but not mutant SENP-1 indicate that the effects seen are not due to noncovalent roles of SUMO. Nonetheless, it is likely that the functional consequences of SUMOylation we observed are mediated by changes in the dynamics of protein interactions. Thus, the SUMOylation-evoked changes will also involve proteins that non-covalently bind SUMO once it has been conjugated to substrate proteins.

Synaptosomes mainly comprise resealed presynaptic buttons and contain many SUMOylated proteins. Using subsynaptic fractionation procedures (Feligioni *et al.*, 2006) we resolved populations of SUMOylated proteins in the presynaptic active zone, the postsynaptic density and in the nonsynaptic synaptosomal fraction. Contrary to our initial expectations, introducing active SUMO into synaptosomes enhanced kainate evoked glutamate release. We have shown that agonist binding to kainate receptors leads to SUMOylation of the GluR6 subunit and subsequent internalisation. We therefore propose that although kainate receptor stimulation (and possible rapid internalisation depending on the subunit composition) initiates the signalling cascade leading to glutamate release it is the SUMOylation of one or more, as yet unidentified, downstream proteins that controls the extent of glutamate release.

The different effects of KCl/AMPA and kainate stimulation suggest that different populations of proteins are differentially SUMOylated, depending on the stimulus, to either enhance or diminish presynaptic release. This observation would imply that there is a complex and compartment-specific SUMO signalling pathways at the synapse that will allow for separate regulation of pre- and postsynaptic proteins. Analysis of these pathways will require identification of both the common and different proteins that are SUMOylated and deSUMOylated in response to stimulation by the different compounds.

An important and intriguing characteristic of SUMO modification is that the biological consequences of conjugation seem out of proportion to the small fraction of substrate conjugated. Transient SUMOylation appears to have the capacity to alter the fate of the substrate even after deconjugation. Thus a non-SUMOylated protein with a prior history of SUMO modification can have different properties from a naive protein that has never been SUMOylated (Hay, 2005). This characteristic may explain, at least in part, why the changes in levels of SUMOylated protein we observed using immunoblotting of synaptosomes containing added SUMO-1 or SENP-1 protein were relatively small.

Because the study of extranuclear and synaptic SUMOylation is in its infancy the mechanisms underlying these different pathways remain to be resolved. For example, active SUMO-GG requires ATP for the initial conjugation step and SUMOylation is regulated by phosphorylation and reactive oxygen species (for reviews see Geiss-Friedlander & Melchior, 2007; Martin *et al.*, 2007b). However, consistent with changes in presynaptic protein SUMOylation, we show that Ubc9, the sole specific SUMO-conjugating enzyme, is enriched in the presynaptic fraction following AMPA stimulation, suggesting activity-dependent recruitment or retention of the SUMOylation enzymes may underlie the enhancement of presynaptic SUMOylation.

One prominent mechanism by which SUMO could modulate neurotransmitter release is by regulating the levels of Ca^{2+} in the presynaptic terminal. We show that altering SUMOylation levels of presynaptic proteins has dramatic effects on calcium entry into synaptosomes, indicating that Ca^{2+} influx is one of the pathways sensitive to SUMO modulation. The large difference between the SUMO-mediated enhancement of glutamate release compared to Ca^{2+} influx is intriguing. There are several possible reasons for this. For example, SUMOylation of proteins at various stages of the release pathway may have additive effects causing an increase in Ca^{2+} influx and then, on top of that, and increase in the sensitivity of release to the raised Ca^{2+} levels. Alternatively, we believe a less probable explanation is that SUMOylation might preferentially affect glutamatergic terminals so the increase in Ca^{2+} influx is averaged across all synaptosomes not only those that release glutamate.

We were initially surprised that inclusion of high levels of purified recombinant wild-type immature SUMO did not modulate evoked glutamate release. In contrast, inclusion of already mature SUMO-1 (GG) had marked effects. SUMO-1 must be matured by cleavage of the last four C-terminal amino acids to expose the diglycine motif required for covalent attachment to the substrate protein. This cleavage event is mediated by the same SENP enzymes that also de-SUMOylate substrate proteins. We therefore attribute the lack of effect of immature SUMO-1 to insufficient levels of endogenous SENP enzymes in the synaptosomes to mature the high concentration of entrapped recombinant SUMO-1.

A key question, which we are not yet in a position to answer, is what are the presynaptic SUMO targets that modulate evoked Ca^{2+} influx and glutamate release from synaptosomes? There are numerous candidate proteins and we anticipate that the combined actions of multiple substrates regulate these processes. We have attempted to identify presynaptic SUMOylated proteins using a 'likely candidate' approach and a bacterial SUMOylation assay system (Uchimura *et al.*, 2004a,b). For example, we tested the Ca^{2+} channel subunit CaV2.2 (Catterall, 2000), vesicle release proteins synaptotagmin II and III (Rizo *et al.*, 2006) and SNAPs (Stenbeck, 1998). Despite the presence of high probability consensus SUMOylation sites none of these proteins showed any indication of SUMOylation in the bacterial assay (Wilkinson *et al.*, 2008) and data not shown.

In summary, the functional and pathophysiological implications for synaptic protein SUMOylation are far-reaching. Our data show that SUMO conjugation to presynaptic target proteins regulates calcium influx and neurotransmitter release. We expect that, similar to other posttranslational modifications such as phosphorylation and ubiquitination, protein SUMOylation will prove to be involved in a diverse array of pathways. Determining the specific SUMO targets, SUMO-binding proteins and precise regulatory mechanisms at the presynaptic terminal and elsewhere represents a new and important challenge.

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Abbreviations

GG	diglycine motif
NEM	<i>N</i> -ethylmaleimide
SENP	SUMO-specific cysteine protease
SUMO	small ubiquitin-like modifier
t-TBS	Tris-buffered saline–Tween

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Fig. 1.

Synaptic SUMO-conjugated proteins and Ubc9 presence in synaptosomes. (A) Immunoblots of total tissue homogenate and synaptosomes prepared with or without the cysteine-alkylating agent NEM (20 mM) to preserve SUMOylation via inhibition of SENP activity. Fifty micrograms of protein was loaded onto each lane of 10% gel, and SUMO-conjugated bands were quantified by densitometry of the entire lane in the range 25–250 kDa. Membranes were stripped and probed for β -tubulin as a loading control. (B) Histogram of data from four experiments expressed as the mean $\pm$ SEM of control (no NEM). Three degrees of freedom, $*P < 0.05$ vs. control, Student's *t*-test. (C) Ubc9 was present in synaptosomes. (D) Subsynaptic redistribution of Ubc9 following AMPA stimulation of synaptosomes. AMPA (50 μ M) was applied for 3 min and synaptic fractionations were prepared in the presence of NEM (20 mM). Twelve micrograms per lane of protein were loaded on 12% gel. (E) Quantified data from four experiments expressed as a percentage of the nonstimulated control condition (mean $\pm$ SEM). Three degrees of freedom, $*P < 0.05$ vs. control, Student's *t*-test. NSSP, nonsynaptic synaptosome protein.

Fig. 2.

Effect of stimulation on protein SUMOylation. Immunoblots of synaptosomes were treated with (A) KCl (30 mM), (B) AMPA (50 μ M), (C) NMDA (50 μ M) or (D) kainate (KA; 10 μ M) for 3 min. Subs synaptic fractions were prepared and 12 μ g/lane of protein was loaded onto 10% gels. NEM (20 mM) was present throughout. SUMO-conjugated bands were quantified by densitometry of the entire lane in the range 75–20 kDa. Histograms show the quantified data from five experiments expressed as the mean $\pm$ SEM of control (no NEM). Four degrees of freedom, * P < 0.05 vs. control, Student's t -test.

Fig. 3.

Effects of SUMO-1 and SENP-1 on KCl- or kainate-evoked glutamate release from synaptosomes. (A) Increased SUMOylation in synaptosomes loaded with exogenous SUMO-1(GG) and decreased SUMOylation with SENP-1. (B) Representative experiments illustrating the effects of KCl on glutamate release (30 mM, 3 min) from synaptosomes containing SENP-1. The symbols denote □, wt-SENTP-1 (100 nM), ▲, inactive SENTP-1 mutant (100 nM) and ●, control (no entrapped proteins). (C) Quantified data from nine experiments. * $P < 0.05$ vs. control. (D) KCl (30 mM, 3 min) evoked glutamate release from synaptosomes containing: ◆, wild-type immature SUMO-1 (100 nM), □, SUMO-1(GG) (100 nM), ▲, SUMO-1(ΔGG) (100 nM) and ●, control. (E) Quantified data from seven experiments. * $P < 0.05$ vs. control. (F) Kainate stimulation (10 μ M, 3 min) of synaptosomes containing SENP-1, and (G) quantified data from five experiments. (H) Kainate stimulation of synaptosomes containing SUMO-1, and (I) Quantified data from six experiments. * $P < 0.05$ vs. control. Where required two-way ANOVA followed by Bonferroni's test were performed.

Fig. 4.

Effects of SUMO-1 and SENP-1 on Ca^{2+} influx. Synaptosomes containing SENP-1 or SUMO-1 were treated with: (A and B) KCl (30 mM); (C and D) AMPA (50 μ M); (E and F) NMDA (50 μ M); and (G and H) kainate (10 μ M) for 3 min. Ca^{2+} was measured using Fura-2AM dye. Quantified data from at least five experiments per condition expressed as a percentage of control (mean $\pm$ SEM). Statistical analysis was performed with two-way ANOVA, followed by Bonferroni's test. * $P < 0.05$ vs. control.