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Analysis of SUMO-1 modification of neuronal proteins containing consensus SUMOylation motifs

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ABSTRACT

SUMOylation is emerging as an important mechanism for modulating protein function in many cell types. A large variety of proteins have been proposed as SUMO targets based on the presence of a consensus SUMOylation core motif (Ψ-K-x-D/E). In neurons these include multiple synaptic proteins but it has not been established whether proteins carrying this motif are SUMOylated either *in vitro* or *in vivo*. Here we use a bacterial SUMOylation assay to systematically test for SUMO-1 modification of a selection of neuronal proteins containing one or more amino acid sequences predicted as high-probability SUMOylation sites in computer-based searches. Of the 39 proteins analysed only 14 sites were posttranslationally modified by SUMO-1, including the group III metabotropic glutamate receptors and the kainate receptor subunit GluR7. These results identify new candidate proteins that may be involved in the SUMO regulation of synaptic activity and also demonstrate that the presence of the Ψ-K-x-D/E motif is not sufficient to indicate that a protein can be SUMOylated in this bacterial system.

SUMO is an ~11 kDa protein which is conjugated to lysine residues in substrate proteins in an enzymatic cascade analogous to that of the ubiquitin pathway [15]. Yeast contain only one SUMO protein, Smt3, whereas mammals possess 4 SUMO isoforms, designated SUMO-1 to SUMO-4 [2]. SUMO-1 shares ~18% homology with ubiquitin, however, despite the low sequence homology, SUMO proteins have a very similar three-dimensional structure to ubiquitin [1,21]. The SUMO proteins can be classed into two subfamilies – SUMO-1 and SUMO-2/3. In their conjugatable forms, SUMO-2 and -3 differ only in 3 N-terminal residues and have yet to be functionally distinguished (and are therefore often collectively referred to as SUMO-2/3), however they only share ~50% homology with SUMO-1 [10,17]. The role of SUMO-4 is unclear since it does not appear to be conjugated to substrate proteins due to a proline residue which prevents maturation of the immature polypeptide [24].

SUMO is conjugated to target proteins via four sequential enzymatic steps. The four C-terminal residues are cleaved from nascent SUMO-1 by members of the SENP family of proteins, exposing a di-glycine motif which is conjugated to target proteins. This conjugatable SUMO is then activated in an ATP-dependent manner by the E1 enzyme, a dimer of SAE1/SAE2, and passed to the active-site cysteine of the E2 'conjugating' enzyme Ubc9 [10,15,21]. In many cases, Ubc9 is sufficient for the transfer of activated SUMO to target proteins, however a number of E3 'ligase' proteins have been described, which facilitate transfer of SUMO from Ubc9 to the substrate *in vivo*. However, each of the described E3 enzymes appears to act by binding Ubc9 and/or the substrate, bringing them into a position more conducive to SUMO transfer. Thus, the actual transfer of SUMO to the substrate is always dependent on Ubc9. Because of this, SUMOylation generally occurs in a consensus motif, which is directly bound by Ubc9 [10,29]. This consensus motif can be described as Ψ-K-x-D/E, where Ψ is a large hydrophobic residue, K is the target lysine, *x* can be any residue, and D/E are aspartate or glutamic acid (acidic residues).

Several computer algorithms have been designed that search protein sequences for potential SUMOylation sites. However, these are limited since the consensus sequence contains only four residues and is relatively degenerate. For example, of 5884 open reading frames (ORFs) in *Saccharomyces cerevisiae*, there are 2799 occurrences of the motif [IVL]-K-x-E in 1913 ORFs [15]. Thus, although it has not been established experimentally, only a small proportion of these 'hits' are likely to be *bona fide* SUMO substrates. Attempts to improve SUMO site prediction have focused on defining extended SUMOylation consensus motifs. The negatively-charged SUMOylation motif (NDSM) is based on the observation that many SUMO substrates contain an acidic patch of amino acids downstream of the Ψ-K-x-D/E core motif that interacts with a corresponding basic patch on Ubc9 [36]. Similar to the NDSM, the phosphorylation-dependent SUMOylation motif (PDSM) is defined by a phosphorylated serine residue five residues downstream of

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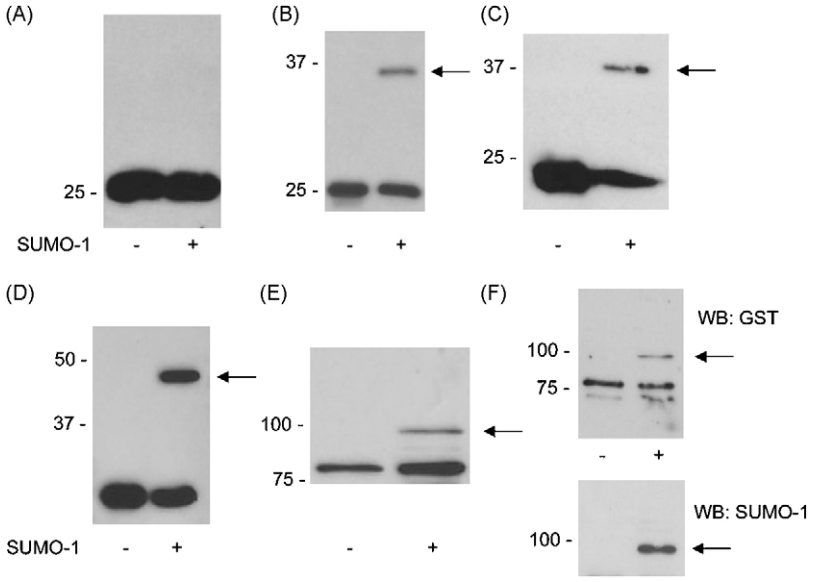

Fig. 1. Efficient SUMOylation of known substrate proteins. Vectors encoding GST, or known SUMOylation substrates were transformed into bacteria with or without the SUMOylation plasmid (indicated by '+' or '−'). Crude bacterial lysates were then subjected to Western blotting for GST or 6×His, as appropriate. SUMOylated species are indicated by an arrow. (A) GST alone, (B) GST-tagged PML (residues 485–495), (C) 6×His-tagged RanGAP (entire C2 domain), (D) GST-tagged GluR6a (intracellular C-terminus), (E) GST-p53 (full length), (F) GST-p53 was purified on glutathione beads and probed for GST (upper panel) or SUMO-1 (lower panel), confirming the band shift seen in (E) is due to SUMOylation of p53.

the Ψ-K-x-D/E motif [13]. In this case rather than being encoded in the primary protein sequence, the acidic patch is provided by phosphorylation of the downstream serine that can act as a signal for SUMOylation.

Recently, as part of a study of SUMOylation of the kainate receptor subunit GluR6 we showed that there are multiple, as yet unidentified synaptic SUMO substrates [20]. Furthermore, together with others we have shown that ischemia causes a massive upregulation of protein SUMOylation in brain that may a represent a neuroprotective mechanism [4,19,37]. A key question, therefore, is what proteins are targets for SUMOylation in neurons. To identify potential synaptic SUMO substrates we performed a bioinformatic screen of candidate synaptic proteins for high-probability SUMOylation sites using SUMOplot, and the NDSM motif. We then directly tested proteins that scored highly in these screens in biochemical SUMOylation assays. Because immunoprecipitation of SUMOylated proteins from mammalian cells is particularly challenging due to the very low levels of SUMO modification and the extensive deSUMOylation activity (for reviews see [10,15,21]), we used a recombinant bacterial SUMOylation assay [33,34]. In all we tested 39 proteins with a total of 58 high-probability SUMOylation sequences including 4 NDSMs. Our data show that the presence of consensus sequences is a relatively poor indicator of actual protein SUMOylation in this assay system.

Plasmid Constructs: pE1E2S1, a bacterial expression vector containing a fusion of SAE1/2, Ubc9 and SUMO-1 [33,34] was obtained from Dr Hisato Saitoh (Kumamoto University, Japan). GST-tagged bacterial expression vector receptor C-termini and GST-syntenin were generated by Dr Helene Hirbec while in our lab. GST-fusions of synaptotagmin II and III were from Dr Gianpietro Schiavo (Cancer Research UK, London, UK), GST-fusions of sorting nexins 1, 2, 4 and 27 were from Dr Peter Cullen (University of Bristol), GST-fusion of full-length p53 was from Dr Kevin Gaston (University of Bristol), 6×His-tagged β-2-adaptin was from Dr Tomas Kirchhausen (Harvard Medical School, MA, USA) and GST-tagged CaV2.2 I-II linker region was from Dr Annette Dolphin (University College London, UK).

Antibodies: The sources and dilutions of antibodies were; goat polyclonal anti-GST (GE Healthcare: 1:1000), rabbit polycolonal anti-β-2-adaptin (Santa Cruz, 1:200) rabbit polyclonal anti-SUMO-1 (Santa Cruz, 1:200), mouse monoclonal anti-6×His (Roche, 1:1000) mouse monoclonal anti-CamKIIα (Santa Cruz, 1:200), mouse monoclonal anti-HA-tag antibody (Santa Cruz, 1:200) and HRP-conjugated anti-mouse, anti-goat or anti-rabbit antibodies (Sigma, 1:10000).

Bacterial SUMOylation Assay: The bacterial SUMOylation assay was performed as described previously [33].

Purification of GST-tagged Proteins: One millilitre of induced bacterial culture was spun down at 16,000 × *g* for 1 min and the supernatant discarded. To each pellet, 0.5 ml lysis buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 20 mM NEM, 1% (v/v) Triton X-100) was added, and the cells lysed by sonication. Insoluble material was removed by spinning at 16,000 × *g* for 10 min and the supernatant collected. To each supernatant, 2 μl 2-β-marcaptoethanol was added to neutralise unreacted NEM, which could interfere with GST binding to glutathione by alkylation of the cysteine residue of glutathione. Thirty microlitres (bed volume) of glutathione sepharose (Sigma) was added and the sample incubated on a rotating wheel at 4 °C for 30 min. Beads were then washed four times with PBS. Proteins were then eluted from the beads by boiling in SDS-PAGE sample buffer.

SDS-PAGE and Western Blotting: Proteins were separated on 6–12% acrylamide gels and transferred onto PVDF membrane (Millipore). Membranes were blocked in TBST containing 5% (w/v) powdered skimmed milk before incubation with the primary antibody diluted in TBST/5% milk for 1–16 h. After washing with TBST, membranes were incubated with secondary antibodies diluted in TBST/5% milk for 1 h and after further extensive washing in TBST, developed with enhanced chemiluminescence substrate (Roche).

The presence of E1, E2 and SUMO is the minimal requirement for target protein SUMOylation [23]. E3 ligase enzymes facilitate SUMO transfer *in vivo* but in many cases, are not essential [6,23,27]. In the bacterial SUMOylation assay a single vector encoding E1, E2 and SUMO-1 or SUMO-2 is cotransfromed with a vector expressing the potential SUMO target protein. This system has been validated for multiple known target proteins and it is effective for both short fragments and full-length proteins [34]. It should be noted, however, that the lack of SUMOylation in this bacterial assay does not