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Synthetic derivatives of the SUMO consensus sequence provide a basis for improved substrate recognition

Melissa J. Leyva [a], Yeong Sang Kim [a], Megan L. Peach [b], John S. Schneekloth Jr. [a,*]

[a] Chemical Biology Laboratory, National Cancer Institute, 376 Boyles St., Frederick, MD 21702, USA
[b] Basic Science Program, Chemical Biology Laboratory, Leidos Biomedical Research, Inc., National Cancer Institute, 376 Boyles St., Frederick, MD 21702, USA

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ABSTRACT

Protein sumoylation is a dynamic posttranslational modification that regulates a diverse subset of the proteome. The mechanism by which sumoylation enzymes recognize their cognate substrates is unclear, and the consequences of sumoylation remain difficult to predict. While small molecule probes of the sumoylation process could be valuable for understanding SUMO biology, few small molecules that modulate this process exist. Here, we report the synthesis and evaluation of over 600 oxime-containing peptide sumoylation substrates. Our work demonstrates that higher modification efficiency can be achieved with non-natural side chains that deviate substantially from the consensus site requirement of a hydrophobic substituent. Furthermore, docking studies suggest that these improved substrates mimic binding interactions that are used by other endogenous protein sequences through tertiary interactions. The development of these high efficiency substrates provides key mechanistic insights toward specific recognition of low molecular weight species in the sumoylation pathway.

The posttranslational modification of protein substrates with the small ubiquitin-like modifier (SUMO) is a highly dynamic process critical to many cellular phenomena. Similar to ubiquitin, SUMO utilizes a cascade of E1, E2, and E3 enzymes to catalyze covalent conjugation to protein substrates.[1–3] In addition to being required for cell cycle progression and development,[4] DNA damage repair,[5] survival of heat shock,[6] and protection from ischemia,[7] this pathway is dysregulated in a number of cancers.[8–10] A growing number of proteins within the genome have been identified as substrates for SUMO-1, -2, and/or -3 modification,[11,12] and the consequences of this modification are currently an area of significant investigation. Although great strides have been made in structural biology, the specific mechanism by which so many structurally diverse substrates within the proteome are recognized by sumoylation enzymes remains in question. The biological significance of this conjugation pathway emphasizes the need for new methods to interrogate molecular recognition events of sumoylating enzymes.

One unique aspect that differentiates the sumoylation pathway from other ubiquitin-like signaling pathways is that Ubc9, the SUMO E2 enzyme, directly recognizes a substrate consensus sequence.[13] This sequence is typically described as ψKxE, where ψ is a hydrophobic amino acid, K is the lysine where the SUMO isopeptide bond is formed, x is any amino acid, and E is an acidic amino acid. This sequence is found in a broad variety of proteins, such as IκBα, p53, PML, and AdE1B, where it typically occurs in exposed flexible loops. In addition to this canonical sequence, several variant consensus sequences have been found, including inverted sequences,[4] hydrophobic clusters,[14] negatively charged consensus motifs,[15] and phospho-dependent sumoylation sequences.[16] These short sequences provide at least one basis for specificity in substrate recognition in the sumoylation pathway. We reasoned that development of a small, more efficient substrate might provide insight into the molecular basis of substrate recognition by Ubc9, or serve as a starting point for the identification of small molecule ligands for the enzyme. To the best of our knowledge, these efforts are the first attempts to derivatize the sumoylation consensus sequence in order to identify substrates with improved efficiency, though one synthetic substrate has been serendipitously identified via a screening approach.[17]

Here, we report the identification of small, synthetic substrates that undergo SUMO modification with superior efficiency than endogenous peptide sequences. We utilize an oxime scanning approach to prepare and evaluate 600 chemically diverse oxime-containing non-natural peptide sequences as substrates for sumoylation (Fig. 1). Two of these peptides were found to be significantly more reactive than the native peptide sequence itself, and were studied in further detail. In silico modeling experiments provide

* Corresponding author. Tel.: +1 301 228 4620; fax: +1 0301 846 6033.
E-mail address: schneeklothjs@mail.nih.gov (J.S. Schneekloth).

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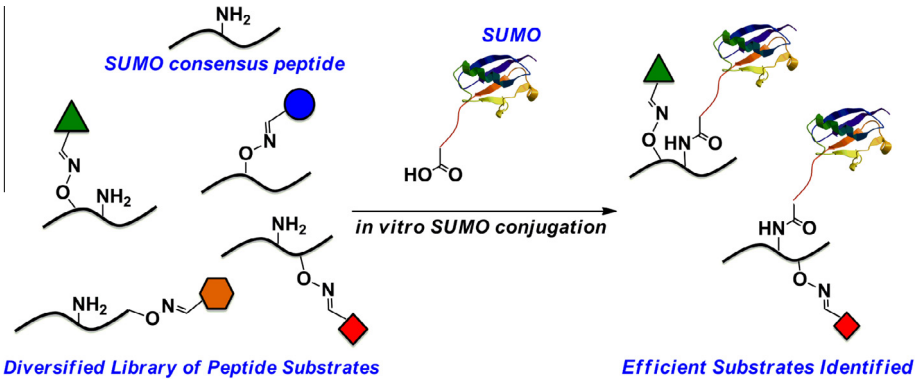

Figure 1. Schematic representation of an oxime scanning strategy to identify highly active sumoylation consensus peptide mimics.

a rationale for the improved recognition of these substrates relative to natural sequences.

We previously reported the use of a 10-mer peptide derived from the androgen receptor and labeled with the fluorophore 5-FAM (FL-AR) as a competent substrate in a microfluidic electrophoretic mobility shift biochemical assay.[18] It is well known that certain short peptide sequences derived from endogenous substrates will recognize Ubc9 directly and undergo sumoylation in biochemical assays, even in the absence of E3 ligases.[19,20] Generally, these peptides must contain the ψKxE sequence, and substantial deviations from this sequence decrease modification efficiency. To investigate the ability of shorter sequences to undergo sumoylation, we evaluated a series of four AR-derived peptides as competitive substrates in our biochemical assay. This assay utilizes a microfluidic system to separate fluorescently labeled substrate and product of an enzymatic reaction on the basis of differing electrophoretic mobility. Under optimized separation conditions, both the substrate FL-AR peptide and sumoylated product could be observed. Thus, percent conversion could be quantified ratiometrically. In this study, we measured an IC_{50} for each peptide for inhibition of FL-AR sumoylation in order to compare substrate efficiencies (Fig. 2A). Thus, each peptide was evaluated for its ability to outcompete sumoylation of the fluorescent FL-AR peptide substrate. In this assay the AR-derived 10-mer, 8-mer, 6-mer, and 4-mer peptides were active as competitive substrates, with IC_{50} values of 9 ± 0.1, 6 ± 0.2, 8 ± 0.3, and 30 ± 7 μM, respectively. It is notable that the simple tetrapeptide, composed of the consensus sequence alone, was competent in the assay, albeit slightly less so than the longer sequences.

Having established that the AR-derived tetrapeptide is a competent substrate for SUMO modification, we evaluated five other tetrapeptide sequences from known sumoylation substrates. We synthesized and evaluated sequences from RanGap1, IκBα, p53, PML, and AdE1B as competitive substrates in dose response (Fig. 2B).[13] Here, Ac-IKLE-NH_2 (the sequence from AR) and Ac-IKME-NH_2 (the sequence from PML) were the most active peptides, with an IC_{50} of 30 ± 7 μM and 33 ± 2 μM, respectively. On the basis of these experiments, we elected to pursue Ac-IKLE-NH_2 as the scaffold on which to synthesize our library.

In order to rapidly investigate large numbers of modified peptides related to the SUMO consensus sequence as competitive substrates, we utilized an oxime scanning approach. Oxime bond ligation has shown to be a useful strategy to rapidly generate a library of compounds.[21–30] Some of the advantages of using oxime ligation over other click reactions are that it can be conducted using commercially available aldehydes and reaction products can be directly biologically evaluated without purification. Six different peptides were prepared by solid phase peptide synthesis, each of which contained an aminooxy (AO) group

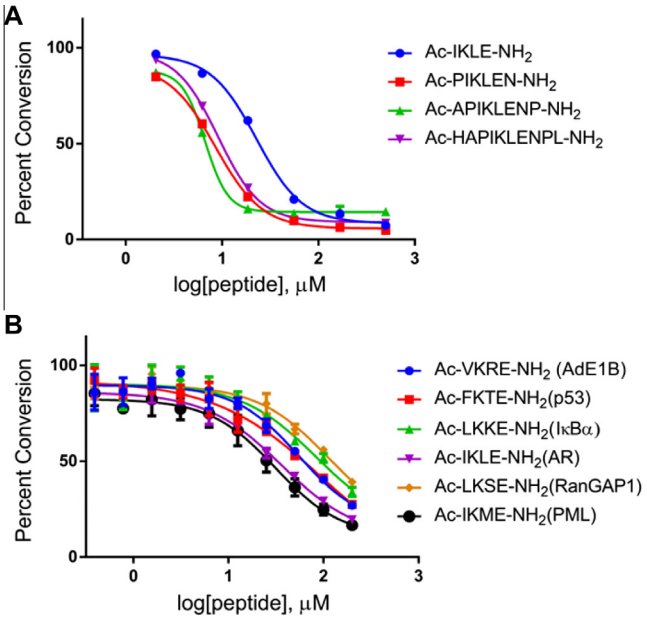

Figure 2. Assessment of synthetic polypeptides as competitive sumoylation substrates in a microfluidic electrophoretic mobility shift biochemical assay. Effects of (A) varied peptide length and (B) varied sequence (six consensus sequences from endogenous sumoylation substrates). All peptides were prepared by solid phase peptide synthesis, are acetylated at the N-terminus, and amidated at the C-terminus. Error bars represent 1 standard deviation from the mean ($n = 3$). When error bars are not visible, they are smaller than the size of the symbols. Percent conversion is normalized to DMSO control.

replacing a different residue in the consensus sequence or at the C-terminus of the peptide (Fig. 3A). Each peptide was then condensed with a chemically diverse library of 100 aldehydes to generate a library of 600 SUMO consensus sequence mimetics. Each oxime-containing peptide was then individually evaluated at a concentration of 25 μM in our previously reported biochemical sumoylation assay.

The results of this screen revealed that substrate efficiency depends to a striking extent on the identity of the modified residue. The vast majority of the 600 assayed peptides were substantially less active than the consensus sequence-containing peptide itself, highlighting the specificity required for Ubc9 to recognize its substrates (Fig. S1 in Supporting information). Only 10 substrates derivatized at the ψ- and C-terminal positions displayed comparable or improved activity relative to Ac-IKLE-NH_2. These compounds were further evaluated after resynthesis and purification but only a few gave reproducible results. From this analysis, we identified compounds **3** and **4** as the most active peptides