
Enhanced protein expression in mammalian cells using engineered SUMO fusions: Secreted phospholipase A₂

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(RECEIVED April 3, 2008; FINAL REVISION June 4, 2008; ACCEPTED June 4, 2008)

Abstract

SUMOylation, the covalent attachment of SUMO (small ubiquitin-like modifier), is a eukaryotic post-translational event that has been demonstrated to play a critical role in several biological processes. When used as an N-terminal tag or fusion partner, SUMO has been shown to enhance functional protein production significantly by improving folding, solubility, and stability. We have engineered several SUMOs and, through their fusion, developed a system for enhancing the expression and secretion of complex proteins. To demonstrate the fidelity of this fusion technology, secreted phospholipase A₂ proteins (sPLA₂) were produced using HEK-293T and CHO-K1 cells. Five mouse sPLA₂ homologs were expressed and secreted in mammalian cell cultures using SUMO or SUMO-derived, N-terminal fusion partners. Mean and median increases of 43- and 18-fold, respectively, were obtained using novel SUMO mutants that are resistant to digestion by endogenous deSUMOylases.

Keywords: SUMO; SUMO fusion; phospholipase A₂; mammalian expression; deSUMOylase

SUMO (Small Ubiquitin-like MODifier) and the SUMO pathway are highly conserved throughout eukaryotes. SUMOylation of proteins in humans was discovered recently by Blobel and colleagues in studies of protein compartmentalization (Matunis et al. 1996). Although SUMO has low (18%) sequence identity with ubiquitin, the two proteins are very similar structurally. The SUMO and ubiquitin pathways also share many features, for instance, both proteins are synthesized as precursors requiring a proteolytic processing step prior to target protein conjugation, and each has a series of activating, conjugating, and ligating enzymes that attach the C termini of the respective proteins to the ϵ -amino group

of lysines in the target proteins (Johnson 2004). SUMO and ubiquitin hydrolases both play important regulatory roles by their removal of SUMO or ubiquitin from target proteins; however, the mechanistic similarity ends at the fate of the modified protein. Unlike ubiquitin, SUMO is not a signal for proteolysis by the 26S proteasome. Although SUMOylation is recognized as an important regulatory modification, its precise role is still the subject of active study. Several papers implicate SUMOylation as a translocation signal for nuclear-cytosolic trafficking. Paradoxically, lysine residues in target proteins that are SUMOylated are protected from ubiquitylation, while SUMOylation of proteins can also serve as a signal for ubiquitylation (Prudden et al. 2007). Thus, nature has evolved two pathways that have remarkably similar mechanical and biochemical features, yet have very distinct roles in cellular physiology (Johnson 2004).

Fusion to SUMO has been shown to enhance the expression of recombinant proteins in *Escherichia coli* (Malakhov et al. 2004; Butt et al. 2005; Zuo et al. 2005; Marblestone et al. 2006; Panavas et al. 2008), while fusion to hSUMO3 led to similar enhancement in *E. coli* (data not shown) and *Pichia pastoris* (R.J. Peroutka, D. Roy, J. Strickler, and T.R. Butt, in prep.). Based on the original observation of

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Abbreviations: CHO-K1, Chinese hamster ovary; HEK-293T, human embryonic kidney cells; sPLA₂, secretory phospholipase A₂; SUMO, small ubiquitin-like modifier; CTHS, C-terminal half SUMO; GFP, green fluorescent protein; NBD C₆-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.035576.108>.

split-ubiquitin (Johnsson and Varshavsky 1994), C-terminal half SUMO (CTHS) was developed for baculovirus/insect cell expression, such that it could be removed only in the presence of its N-terminal half (NTHS). We found that CTHS fusion enhanced the production of fusion partners in insect cells while avoiding endogenous cleavage (data not shown). SUMOstar was developed with the same goal—creating a SUMO fusion, which in a eukaryotic host would not be cleaved *in vivo*, but would undergo enhanced expression similar to the enhancement demonstrated in prokaryotes. Following crystal structure analysis of SUMO bound to its natural protease Ulp1 (PDB: 1EUV), a rational mutagenesis screening campaign resulted in the modification of two interfacial amino acids. These modifications, R64T and R71E, resulted in a SUMO that was resistant to cleavage by Ulp1 regardless of enzyme concentration. This novel SUMO, termed SUMOstar, displayed an enhancement in the expression of its fusion partner in prokaryotes equivalent to that obtained with wild-type SUMO (data not shown); however, it was not clear whether SUMOstar would be cleaved by mammalian deSUMOylases.

To determine (1) if the novel SUMO was equally resistant to mammalian deSUMOylases and (2) whether a similar enhancement could be accomplished in mammalian cells, we examined the effect of various SUMOs on the expression of several members of the secreted phospholipase A₂ family (sPLA₂) in human embryonic kidney (HEK-293T) and Chinese hamster ovary (CHO-K1) cells.

The sPLA₂ family was selected for expression studies because these enzymes, like many complex proteins, have traditionally been expressed in *E. coli* as inclusion bodies and refolded for characterization. In addition, a native N terminus is required for accurate biological activity, which can be quantified by any of a number of facile assays. Secretory PLA₂ was first discovered in cobra venom in the 1890s, but most sPLA₂s have been discovered only within the past two decades (for review, see Six and Dennis 2000; Schaloske and Dennis 2006). PLA₂s are named for their specific cleavage of phospholipids at the *sn*-2 acyl bond yielding free fatty acids, such as arachidonic acid (AA), and lysophospholipids (lyso PL). Both products of PLA₂ digestion are precursors of a variety of signaling molecules involved in diverse biological pathways. To date, five principal PLA₂ families have been identified, with enzyme assignment based on specific function, method of function, and structure (Schaloske and Dennis 2006). The sPLA₂ family is comprised of enzymes that are relatively small in size (12–18 kDa), possess five to eight disulfide bonds, contain active sites with a His/Asp dyad, and require μM Ca²⁺ for activity. To date, 10 sPLA₂s have been identified in humans and 11 in mice, exhibiting various functions and tissue distribution. Mammalian sPLA₂s have been implicated in a wide range of diseases such as asthma (Henderson Jr. et al. 2007), atherosclerosis (Webb 2005), and cancer (Murakami et al. 2005). Biological relevance is not

limited to enzymatic activity, as sPLA₂s have been shown to act as ligands themselves, interacting with at least two receptors in mammals (Lambeau and Lazdunski 1999). Of the 11 mouse sPLA₂s, groups IIC, IIE, III, V, and X were selected for the present study because they cover the widest range of expression levels in prokaryotes, ~0.8 to 20 mg/L (Rouault et al. 2007).

Our results show that: (1) Native SUMO fusions can be processed in mammalian cell culture; (2) when overexpressed, and released from their fusion partner, some sPLA₂s exhibit moderate toxicity; and (3) fusion to SUMO mutants, not recognized by endogenous deSUMOylases, can lead to significantly enhanced expression of sPLA₂s while avoiding cell toxicity. We believe that the development of these mutant SUMOs and their related enzymes will provide valuable tools for enhancing the expression and secretion of difficult to express recombinant proteins in eukaryotic cells.

Results

Development of novel SUMO tags

To evaluate the potential utility of expressing SUMO-fusion proteins in the mammalian secretory pathway, mouse sPLA₂-X was used as a model protein. Initially, the following four N-terminal fusions were tested: SUMO (SUMO), the C-terminal half of SUMO comprising amino acids 55–98 (CTHS), a R64T R71E double mutant SUMO (SUMOstar), and human SUMO-3 (hSUMO3). All tags were created with a hexahistidine (6xHis) N terminus and directed for secretion using the IgG kappa secretory signal from mouse. For control purposes, a vector was created with only the signal sequence and 6xHis tag, creating a total of five vectors differing only in their SUMO-based tag.

Expression and secretion of sPLA₂-X in monolayer HEK-293T cells were examined by Western blot of cell culture media after 48 h (Fig. 1A). SUMOstar showed a significant enhancement in the production of sPLA₂-X compared to expression of the 6xHis or CTHS tag fusions, while no SUMO or hSUMO3 fusion was detected. The SUMO and hSUMO3 cultures appeared less confluent, compared to the others, 48 h post-transfection. These differences in growth rate were quantified by determining viable HEK-293T cell counts at 0, 24, and 48 h post-transfection. Cell counts were compared among SUMO, SUMOstar, and GFP transfections. In addition to growth of GFP-transfected cells as a control, growth of nontransformed cells was measured. Cultures transfected with SUMOstar-sPLA₂-X or GFP displayed growth equivalent to that of nontransfected cells, while SUMO-sPLA₂-X-transfected cultures contained significantly reduced numbers of viable cells (Fig. 2). On average, the cultures transfected with SUMO-sPLA₂-X contained 34% and 28% fewer cells after 24 and 48 h, respectively. To investigate whether any SUMO

or hSUMO3 fused sPLA₂-X was produced, all fusions were purified from medium on a small scale using Ni⁺-NTA resin in batch mode, and the eluates were digested with their respective enzymes. Although SUMO and hSUMO3 fusions were not detected in Western blots, some SUMO-protease-dependent and -independent activity was detected after Ni⁺-NTA purification and assay (Fig. 3A). Presumably, the SUMO-protease-independent activity is the result of release of active sPLA₂ by endogenous deSUMOylases. The lack of 6xHis sPLA₂-X activity in Figure 3A is not indicative of its expression level (~15-fold less than SUMOstar), as the tag did not contain a protease cleavage site. The transfection, purification, and assay of the sPLA₂-X constructs were repeated several times with the same results. To confirm that inactive sPLA₂-X was produced and could be activated by removal of the SUMOstar moiety, we purified the fusion using a Ni⁺-loaded IMAC Sepharose resin and digested the partially purified, imidazole eluted protein with SUMOstar protease (Fig. 3B). The imidazole eluate (E) clearly shows a band with the expected molecular weight of the fusion protein (~34 kD), which is lost following digestion by SUMOstar protease (C) to yield bands with the expected

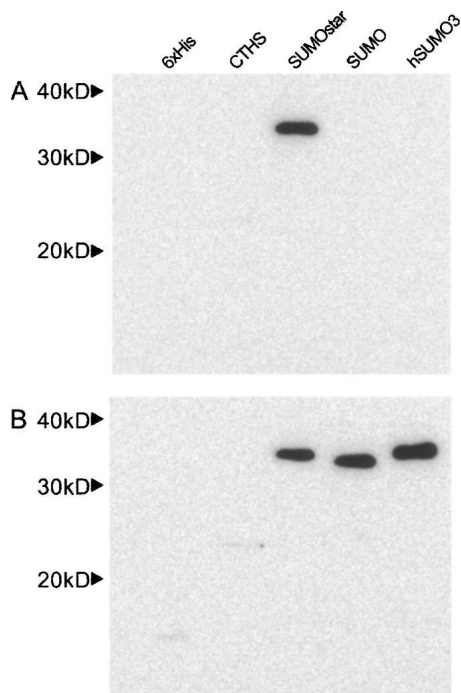


Figure 1. Western blot analyses of sPLA₂-X expression. Samples of media (15 μ L) from mouse sPLA₂-X constructs, 48 h post-transfection (HEK-293T), were separated by SDS-PAGE and transferred to nitrocellulose as described. The blots were probed with a monoclonal anti-6xHis antibody. The following five N-terminal fusion tags were tested: 6xHis, 6xHis-CTHS, 6xHis-SUMOstar, 6xHis-SUMO, and 6xHis-hSUMO3. All constructs were directed for secretion via the mouse IgG kappa secretory signal. Results are representative of at least three independent experiments. (A) Expression of wild-type sPLA₂-X. (B) Expression of Δ Gly-sPLA₂-X.

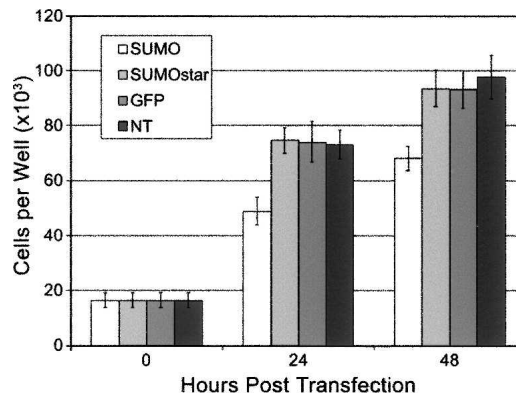


Figure 2. The effect of sPLA₂-X expression on cell growth and viability. HEK-293T cells were seeded in 24-well dishes, and the number of viable cells was determined at 0, 24, and 48 h post-transfection with SUMO-PLA₂, SUMOstar-PLA₂, GFP, or no DNA. Data points represent the average number of viable cells from three independent transfections.

molecular weights of SUMO (~20 kD)¹ and sPLA₂-X (~14 kD). The eluate fraction demonstrated no PLA₂ activity prior to digestion with SUMOstar protease (data not shown).

Mouse sPLA₂-X is naturally produced as a zymogen; its mature form preceded by an 11-amino-acid N-terminal propeptide, which has been observed to reduce activity to 8% (Morioka et al. 2000). The mature form, beginning with GLEEL, was cloned behind the various tags such that fully active PLA₂ would be produced only after *in vitro* release with protease. The recombinant production of active mouse and human sPLA₂-X in mammalian cells has been described, with no mention of toxicity (Cupillard et al. 1997; Morioka et al. 2000), so we did not anticipate the behavior of the SUMO-sPLA₂-X- and hSUMO3-sPLA₂-X-transfected cells. To investigate whether endogenous SUMO cleavage and the resulting phospholipase activity contributed to the differential expression of SUMO and hSUMO3 fusions, compared to SUMOstar, or if the difference was a result of the SUMO proteins themselves, a series of mutant sPLA₂-X fusions was generated. Based on the crystal structure of human sPLA₂-X (PDB: 1LE6), which shares 90% similarity with mouse, we theorized that omitting the N-terminal glycine of sPLA₂-X (Δ Gly-sPLA₂-X) would result in a diglycine that would be less accessible to proteolytic cleavage. Expression of the various fusion tags with Δ Gly-sPLA₂-X in HEK-293T cells can be seen in Figure 1B. By densitometry analysis of 6xHis- and SUMOstar-tagged Δ Gly-sPLA₂-X constructs, SUMOstar led to ~15-fold higher sPLA₂-X production. The results demonstrate that sPLA₂-X activity and the susceptibility of its N-terminal pro-peptide to cleavage clearly play a role in overexpression.

¹SUMO migrates with an apparent molecular weight of 18–20 kDa on SDS-PAGE.

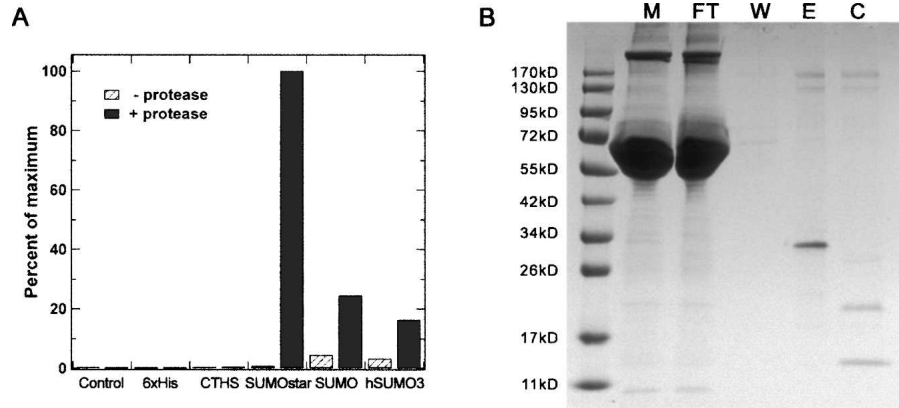


Figure 3. Purification and determination of PLA₂ enzymatic activity. (A) Small-scale Ni⁺-NTA purifications of the initial fusions were performed in batch mode using equal volumes of media, 48 h post-transfection. The eluted samples were digested with the proteases specific for each tag and assayed for sPLA₂ activity, which is represented as a percent of the most active sample. (B) Purification of SUMOstar-sPLA₂-X using a Ni⁺-Sepharose column and subsequent cleavage the fusion with SUMOstar protease. The SUMOstar-sPLA₂ fusion can be seen in the media (M) just below 34 kDa, while it is absent from the flow-through (FT). The column was washed (W), and the fusion (~34 kD) was eluted (E) with imidazole and cleaved (C) with protease, yielding SUMOstar (~20 kD) and free sPLA₂-X (~14 kD).

Expanded expression study

From the initial expression data, we re-evaluated which fusion tags should be tested. We decided that CTHS would not be tested further and that the expression levels of hSUMO-3 with inactive sPLA₂-X warranted further investigation of the human SUMOs. We noticed a strong conservation among SUMOs while evaluating the crystal structures of human SUMO-1, 2, 3, and SUMO; the nearly identical location of two interfacial arginine residues. Since hSUMO-2 and 3 share 97% identity, we elected to investigate only hSUMO-1 and 3. The following modifications were made: for hSUMO1, R63T and R70E; for hSUMO3, R58T and R60E. Wild-type sPLA₂-X and ΔGly-sPLA₂-X fusions were made with the mutant and wild-type versions of SUMO, hSUMO1, and 3. In addition to generating these constructs, a vector containing GFP (without tag or secretory signal) was created for co-transfection purposes. The results of expressing ΔGly-sPLA₂-X and sPLA₂-X fusions for 48 h can be seen in Figure 4, A and B, respectively. Equal amounts of intracellular GFP can be seen in an anti-GFP Western blot below the anti-6xHis blot in Figure 4A, indicating equal transfection efficiencies. The cultures expressing fusions to wild-type hSUMO1, like those expressing SUMO and hSUMO3, displayed limited growth, and therefore less GFP coexpression per culture. Expression of the wild-type forms of SUMO and hSUMO1 could occasionally be detected in replicate culture Western blots, as in Figure 4B, where a faint band of hSUMO1-sPLA₂-X can be seen. Both human SUMO mutants displayed expression comparable to or greater than the original SUMOstar when fused to sPLA₂-X, as seen in Figure 4B. On average, by densitometry analysis of at least three independent transfections, fusions of the three SUMO mutants expressed 15–

20 times more sPLA₂-X than fusions of the 6xHis tag alone. Expression of sPLA₂-X in cultured mammalian cells was further analyzed by the transfection of monolayer CHO-K1 cells. Under the transfection and culture conditions used for HEK-293T cells, CHO-K1 cells displayed very similar expression and secretion after 48 h by Western blot (Fig. 5). It is interesting to note that the CHO-K1 cells appeared to be more sensitive than HEK-293T cells to

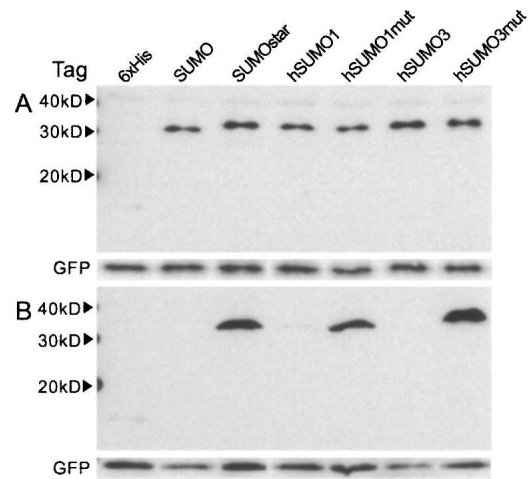


Figure 4. Expression analysis of seven N-terminal fusion tags in HEK-293T cells. (A) Comparison of the ΔGly-sPLA₂-X fusions. All SUMO tags led to an enhancement as compared to the 6xHis tag alone. (B) Expression of wild-type sPLA₂-X fusions. GFP was co-transfected to demonstrate equal transfection. Media samples (15 μL) from the revised mouse sPLA₂-X constructs, 48 h post-transfection (HEK-293T), were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with monoclonal anti-6xHis. Intracellular GFP was detected using polyclonal anti-GFP. Results are representative of at least three independent experiments.

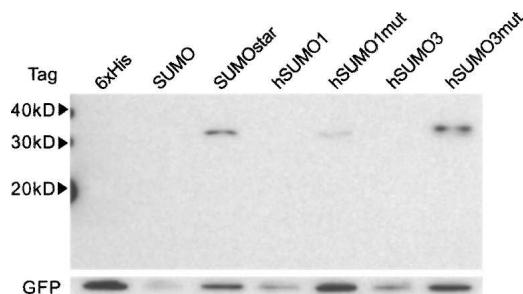


Figure 5. Expression of wild-type sPLA₂-X fusions by CHO-K1 cells. As described in Figure 4, seven N-terminal fusion tags were tested with wild-type sPLA₂-X. Media samples (15 μ L) from the revised mouse sPLA₂-X constructs, 48 h post-transfection (CHO-K1), were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with monoclonal anti-6xHis. Intracellular GFP was detected using polyclonal anti-GFP. Results are representative of three independent experiments.

expression of the natural SUMO fusions, as judged by the confluence of cultures after 48 h and the levels of intracellular GFP.

Given the expression data using mouse sPLA₂-X, we were curious to see whether the enhancements seen in HEK-293T and CHO-K1 production would be comparable using other sPLA₂ groups. We chose four additional mouse sPLA₂ genes based on their varied levels of recombinant expression previously reported (Rouault et al. 2007). Mouse sPLA₂-IIC and III have been produced in insect cells with yields of 150 and 70 μ g/L, respectively. There are currently no refolding protocols for either sPLA₂, and both enzymes are naturally glycosylated, making eukaryotic production a necessity. Mouse sPLA₂-IIE represents the lowest reported yield in bacterial production at 800 μ g/L, while sPLA₂-V represents the highest yield at 20 mg/L. Mouse sPLA₂-X reportedly has been expressed at 10 mg/L in *E. coli*.

We proceeded with the fusion tag comparisons using mouse sPLA₂-IIC, IIE, III, and V in HEK-293T cells. In the interest of time, only the wild-type versions of these enzymes were tested. All transfections were performed with GFP to monitor efficiencies. The intracellular expression of sPLA₂-IIC after 48 h can be seen in Figure 6. His-tagged protein could not be detected in the media. Despite an apparently large increase in expression with all the SUMO tags, secretion was somehow inhibited. The expression and secretion of sPLA₂-IIE can be seen in Figure 7A. After 48 h, significantly more sPLA₂-IIE is visible in most of the SUMO fusions, with 140 times more SUMOstar and 190 times more hSUMO3mut than His-tag alone by densitometry analysis. Mouse and human sPLA₂-III is expressed as a 55-kDa protein but often matures via post-translational and cell-specific proteolytic processing to a 28-kDa active domain (Murakami et al. 2003, 2005). The active or S domain is preceded by an N domain and followed by a

C domain. We generated fusions with the full-length sPLA₂-III, only replacing its native secretory signal with SUMO and the kappa signal. In HEK-293T cells, all sPLA₂-III fusions were apparently processed at their first cleavage point, dividing the N and S domains as seen in Figure 7B, where the His-tagged protein is 12 kDa and the various SUMO fusions are \sim 32 kDa. Intracellular blotting demonstrated the production of a 55-kDa protein with no additional forms visible (data not shown). The expression and secretion of sPLA₂-V can be seen in Figure 7C. Similar to group X, there is a strong preference for the SUMOstar and mutant hSUMO fusions in the expression of sPLA₂-V. Although there was clearly a lack of expression in the fusions to wild-type SUMO, similar cell culture problems were not seen in the case of sPLA₂-V, where, with the exception of hSUMO1, there was little difference in the level of coexpressed GFP. Transfections were performed several times for each sPLA₂ group, using all fusion tags, with comparable results, as analyzed by Western blotting.

Expression levels of the various mouse sPLA₂ fusions were quantified by blotting representative SUMOstar-sPLA₂ samples from each sPLA₂ group, from several transfections with serial dilutions of bacterially expressed SUMO-sPLA₂-X (see Materials and Methods). Although this method is only semiquantitative, it was our best option with such small culture volumes. We found that, on average, SUMOstar-sPLA₂-IIE was produced at 5 ± 0.4 mg/L, while SUMOstar-sPLA₂-III was produced at 4.1 ± 0.1 mg/L. SUMOstar-sPLA₂-V and SUMOstar-sPLA₂-X were, on average, produced at 2.5 ± 1.0 mg/L and 4.3 ± 1.5 mg/L, respectively. The values for the other fusion constructs were extrapolated from the densitometry data of the SUMOstar fusions (Table 1).

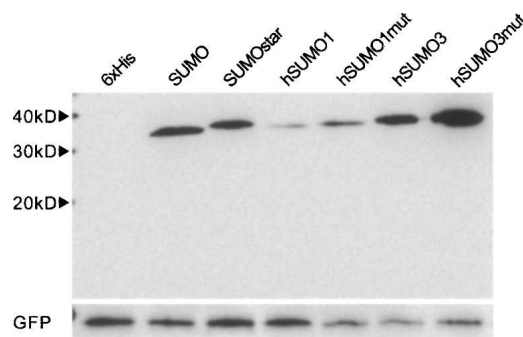


Figure 6. Intracellular expression of sPLA₂-IIC. All constructs being directed for secretion via the mouse IgG kappa secretory signal; however, sPLA₂-IIC was not detected in the medium. Cell lysates, 48 h post-transfection (HEK-293T), were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with monoclonal anti-6xHis. Results are representative of two independent experiments. GFP was co-transfected and detected with polyclonal anti-GFP.

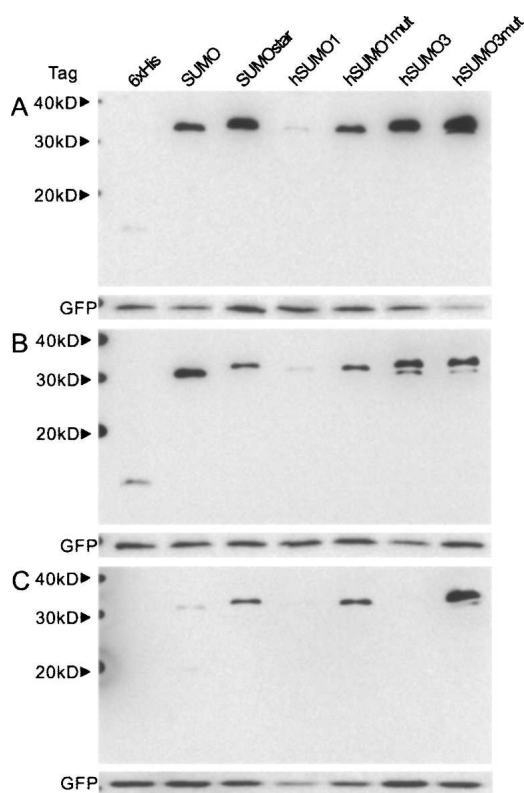


Figure 7. Analysis of N-terminal fusion tags with sPLA₂-IIE, sPLA₂-III, and sPLA₂-V. (A) sPLA₂-IIE samples. (B) sPLA₂-III samples. (C) sPLA₂-V samples. Media samples (15 μ L), 48 h post-transfection (HEK-293T), were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with monoclonal anti-6xHis. Results are representative of two to three independent experiments.

Discussion

Secreted phospholipases were used as model proteins to determine the impact of wild-type and mutant SUMOs on the expression, secretion, and stability of biologically active enzyme. Although mammalian cells contain highly active SUMO proteases, it was not clear whether these enzymes would affect secreted SUMO fusions, as deSUMOylases have not been identified within the secretory pathway. Some, but not all, of the various sPLA₂ fusions were cleaved, indicating an absence of deSUMOylases in the secretory pathway. However, in constructs containing sPLA₂-V and sPLA₂-X, a small amount of presumably extracellular digestion and PLA₂ release was sufficient to elicit a toxic cellular response. This study has shown that, by minor modification, mutant human and yeast SUMO fusions were able to circumvent cleavage in mammalian cell culture while maintaining and/or improving recombinant production levels obtained by wild-type SUMO fusion. Furthermore, phospholipases expressed in large amounts in mammalian cells can be toxic and lead to decreased cell viability. This study has allowed us to make three main

observations. Attachment of a SUMO moiety to the N terminus of target proteins dramatically enhances their expression in cells. Wild-type yeast and human SUMO tags are susceptible to release from their fusion partner, presumably in cell culture media. Toxic proteins such as sPLA₂-X and sPLA₂-V can be produced as dormant fusions, while cleavage of mutant SUMO fusions with mutant SUMO protease *in vitro* generates an active enzyme.

Since they contain large numbers of disulfide bonds, sPLA₂s are difficult to express in *E. coli* and refold following solubilization, typically resulting in poor yields. The recombinant production of secreted mammalian phospholipase A₂s has been established in various hosts including insect cells and larvae (Singer et al. 2002; Zhang et al. 2006), yeast (Lefkowitz et al. 1999; Zhang et al. 2006), chemical synthesis (Hackeng et al. 1997; Dong et al. 2002), mammalian cells (Murakami et al. 1998; Morioka et al. 2000), and *E. coli* (Othman et al. 1996; Singer et al. 2002; Rouault et al. 2006, 2007). Despite the requirement for refolding, the cost of production in *E. coli* is still lower than that in any other system. Many sPLA₂s require a unique refolding procedure, and some of the PLA₂ cannot be produced in active form in *E. coli* (Singer et al. 2002). Therefore, new systems that enable the recombinant production and purification of correctly folded proteins having stringent requirements for defined N termini, and that maintain potentially toxic proteins, such as the secreted phospholipase A₂s, in an inactive state prior to tag removal, would be very useful. We have demonstrated that recombinant fusion of the N terminus of sPLA₂s to the C terminus of yeast and human SUMOs significantly enhances expression

Table 1. Comparison of sPLA₂ (groups IIE, III, V, and X) production from monolayer HEK-293T cells 48 h post-transfection

sPLA ₂	Tag	sPLA ₂ expression (wt tag) (mg/L)	sPLA ₂ expression (mut tag) (mg/L)
mGIIE	6xHis	0.03	
	6xHis-SUMO	2.98	4.98
	6xHis-hSUMO1	0.09	2.11
	6xHis-hSUMO3	4.83	6.00
mGIII	6xHis	1.69	
	6xHis-SUMO	8.16	4.07
	6xHis-hSUMO1	0.32	4.30
	6xHis-hSUMO3	8.71	7.59
mGV	6xHis	0.32	
	6xHis-SUMO	0.50	2.50
	6xHis-hSUMO1	0.89	3.54
	6xHis-hSUMO3	0.90	7.53
mGX	6xHis	0.27	
	6xHis-SUMO	4.29	4.29
	6xHis-hSUMO1	0.06	5.32
	6xHis-hSUMO3	0.01	5.24

and secretion of sPLA₂ proteins in mammalian cell culture. Furthermore, we have developed novel SUMO derivatives that are not processed *in vivo*, and, when fused to sPLA₂ permit overexpression of even the most enzymatically active sPLA₂ without producing toxic effects.

The results of the initial expression comparison using mouse sPLA₂-X were surprising. Despite our efforts to develop a SUMO derivative that was resistant to natural deSUMOylases, we did not expect to see such a difference in the levels of expression among our initial constructs. We theorized, based on previous work with yeast and insect cells, that wild-type yeast SUMO and hSUMO3 fusions would express at levels comparable to those of SUMOstar, but would be at least partially cleaved. In repeated experiments, however, we observed an almost complete lack of recombinant sPLA₂-X expression when fused to SUMO or hSUMO3. Group X is naturally produced as a zymogen with an 11-amino-acid pro-peptide that reduces its enzymatic activity by 92% (Morioka et al. 2000). Since mature sPLA₂-X has been described as possessing a “powerful potency for releasing arachidonic acid” (Morioka et al. 2000), mutant sPLA₂-X fusions were constructed to investigate whether enzyme function was playing a deleterious role in expression. Once sPLA₂-X was mutated, all SUMO-sPLA₂-X fusions displayed significant enhancements in expression (Figs. 1B and 4A).

These observations suggest that uncleaved SUMO fusions can be used as tools to both improve expression of potentially toxic proteins and to study the role of SUMO in cell physiology. Given the high degree of conservation of sequence and structure between SUMOs from different species, it is not surprising that mutation of analogous residues in yeast and human SUMOs generate versions of the proteins that are not recognized or processed by the cognate deSUMOylases. These observations suggest that the SUMO hydrolases recognize similar surfaces of SUMOs in yeast and human cells.

Materials and Methods

Construction of fusion tag vectors

For all vector constructs, pcDNA3.1/V5-His (Invitrogen) was used as a backbone. Platinum Taq DNA Polymerase High Fidelity (Invitrogen) was used for all PCR reactions, while all restriction enzymes and T4 DNA ligase were from Fermentas. Cloning was performed according to standard techniques. All clones were verified by sequencing. All primers used can be found in Table 2. Initially, a kappa S.S. and 6xHis tag were generated via overlapping primers with a region of homology between the two (primers 1 + 2 and 3 + 4, respectively). The kappa S.S and His tag were joined in a secondary PCR reaction using primers 1 + 4. The kappa-6xHis fusion was inserted into pcDNA3.1 via HindIII and XbaI restriction sites, generating

Table 2. Primers used for generating the SUMO vectors and sPLA₂ fusions

	Gene	Sequence	Enzyme(s)	Dir.
1	kappa	GCGCAAGCTTGTCTATGGAGACAGACACTCCTGTATGGGTACTGCTGCTCT	HindIII	F
2	kappa	GATGATGGTGATGACCGTACCAGTGGAACTGGAACCCAGAGCAGTACCCA		R
3	6xHis	CCAGGTTCCACTGGTGACGGTCATCACCATCATCATCAGGAGGT		F
4	6xHis	CGCGTCTAGAGAGACGGCATGCCGTCTCAACCTCCGTGATGATGATGGTGATG	XbaI, Esp3I	R
5	CTHS	CGCAGGTCTCTAGGTGAAAGACAGGGTAAGGAAATGGA	Eco3I1	F
6	SUMO	CGCGTCTAGAGAGACGGCATGCCGTCTCAACCTCCAATCTGTTTCGGGTGA	XbaI, Esp3I	R
7	SUMO	CGCAGGTCTCTAGGTTCCGACTCAGAAGTCAATCAAGA	Eco3I1	F
8	hSUMO3	CGCAGGTCTCTAGGTTCCGAGGAGAAGCCCAAGGA	Eco3I1	F
9	hSUMO3	CGCGTCTAGAGAGACGGCATGCCGTCTCAACCTCCCCTGCTGCTGGAA	XbaI, Esp3I	R
10	sPLA ₂ -X	ATCACGTCTCGAGGTGGACTCCTGGAGCTGGCAGGGAC	Esp3I	F
11	sPLA ₂ -X	GCATCGTCTCACTAGATCAATTGCACTTGGGAGAGT	Esp3I	R
12	ΔGly-PLA ₂ -X	ATCACGTCTCGAGGTCTCCTGGAGCTGGCAGGGAC	Esp3I	F
13	hSUMO1	CGCAGGTCTCTAGGTTCTGACCAGGAGGCCAAAACCT	Eco3I1	F
14	hSUMO1	CGCGTCTAGAGAGACGGCATGCCGTCTCAACCTCCCCTTGTTCCTGATAA	XbaI, Esp3I	R
15	hSUMO1mut	ATGATTATCAGCAATTTCTGACCCTCAAAGAGAAAACGTGAGTGAATTCATTGGAA		R
16	hSUMO1mut	CCAATGAATCACTCACGTTTCTTTGAGGGTCAGGAAATGCTGATAATCATA		F
17	hSUMO3mut	TGGGTGCCCCGTCGAACTCGAATGTGATCTGCCTCATTGACA		R
18	hSUMO3mut	TCAATGAGGCAGATCACATTCGAGTTCGACGGGCAGCCAAT		F
19	sPLA ₂ -IIC	GCGCCGTCTCTAGGTTCTGAGTTTCTGGCAGTTCAGAGGA	Esp3I	F
20	sPLA ₂ -IIC	GCGCCGTCTCTAGATTAGCACTGGAGTTTGTCCCTGC	Esp3I	R
21	sPLA ₂ -IIE	GCGCCGTCTCTAGGTTAACTGGTCCAGTTTGGAGTGA	Eco3I1	F
22	sPLA ₂ -IIE	GCGCCGTCTCTAGATTAGCAGGGTGGGGTGGGC	Eco3I1	R
23	sPLA ₂ -III	GCGCGAAGACATAGGTGCTCACTGGGACAGTACCTCTCTG	BpiI	F
24	sPLA ₂ -III	GCGCGAAGACATAGATTATGAGTCCAGAAATTTCTGTCTG	BpiI	R
25	sPLA ₂ -V	GCGCCGTCTCTAGGTTGGCTTGCTAGACTCAAGTCCATG	Esp3I	F
26	sPLA ₂ -V	GCGCCGTCTCTAGATTAGCAGAGGAAGTTGGGGTAATAC	Esp3I	R

pcDNA3.1-kappa-6xHis (Fig. 8A). Primers 3 and 4 were designed so that the His tag was followed by two glycines and an Esp3I/BsmBI restriction site on the non-coding strand, upstream of the XbaI site. Between the Esp3I/BsmBI and XbaI sites was an additional Esp3I/BsmBI site on the coding strand. Digestion with Esp3I was designed to generate a 4-base overhang on the non-coding strand consisting of TCCA from the di-glycine GGAGGT coding sequence and an XbaI-compatible CTAG overhang on the opposite end of the vector. A single-enzyme, directional double digest was therefore accomplished. CTHS, SUMO, SUMOstar, and hSUMO3 were amplified with primers 5 + 6, 7 + 6, 7 + 6, and 8 + 9, respectively. All reverse primers were designed to recreate the Esp3I recognition sites downstream from the various SUMO terminal di-glycine codons (Fig. 8B). SUMO tags were inserted into pcDNA3.1-kappa-6xHis via Eco31I and XbaI restriction sites generating the following vectors: pcDNA3.1-kappa-6xHis-CTHS, pcDNA3.1-kappa-6xHis-SUMO, pcDNA3.1-kappa-6xHis-SUMOstar, and pcDNA3.1-kappa-6xHis-hSUMO3 (Fig. 8C).

Initial mouse sPLA₂-X construct creation

Active sPLA₂-X was PCR amplified from a clone kindly provided by Dr. Michael Gelb's laboratory (University of Washington, Seattle) using primers 10 + 11 (Fig. 8D). ΔGly-sPLA₂-X was PCR-amplified from the same clone using primers 12 + 11. Both active and inactive sPLA₂-X constructs were created by digesting both PCR product and vectors with Esp3I and ligating digestion products (Fig. 8E).

Expansion of fusion tag vectors

Human SUMO-1 was PCR-amplified from cDNA obtained from Open Biosystems using primers 13 + 14, and cloned into pcDNA3.1-kappa-6xHis via Esp3I and XbaI restriction sites gen-

erating pcDNA3.1-kappa-6xHis-hSUMO1. Mutant human SUMO-1 and 3 were generated using PCR site-directed mutagenesis in which the N-terminal and C-terminal halves were produced in separate reactions, gel isolated, and joined in a subsequent PCR reaction. Human SUMO-1 primary PCR used primers 13 + 15 and 16 + 14 for the N- and C-terminal reactions, respectively. Human SUMO-3 primary PCR used primers 8 + 17 and 18 + 9 for the N- and C-terminal reactions, respectively. In the secondary PCR-purified primary products were mixed for each human SUMO, and primers 13 + 14 were used for hSUMO1mut, while primers 8 + 9 were used for hSUMO3mut. Products were inserted into pcDNA3.1-kappa-6xHis generating pcDNA3.1-kappa-6xHis-hSUMO1mut and pcDNA3.1-kappa-6xHis-hSUMO3mut.

Expansion of mouse sPLA₂ constructs

cDNAs for mouse sPLA₂-IIC, IIE, III, and V were purchased from Open Biosystems. PLA₂ primers were designed with the goal of generating mature proteins subsequent to purification and tag removal. Secretory signals and propeptides were therefore omitted in primer design, based on literature review and SignalP analysis (Bendtsen et al. 2004). Mouse sPLA₂-IIC was cloned from cDNA, corresponding to GenBank entry BC029347, with primers 19 + 20. Mouse sPLA₂-IIE was cloned from cDNA, corresponding to GenBank entry BC027524, with primers 21 + 22. Full-length mouse sPLA₂-III was cloned from cDNA, corresponding to GenBank entry BC079556, with primers 23 + 24. Mouse sPLA₂-V was cloned from cDNA, corresponding to GenBank entry BC030899, with primers 25 + 26. All sPLA₂ genes including sPLA₂-X active and inactive were subcloned into pcDNA3.1-kappa-6xHis, pcDNA3.1-kappa-6xHis-SUMO, pcDNA3.1-kappa-6xHis-SUMOstar, pcDNA3.1-kappa-6xHis-hSUMO1, pcDNA3.1-kappa-6xHis-hSUMO1mut, pcDNA3.1-kappa-6xHis-hSUMO3, and pcDNA3.1-kappa-6xHis-hSUMO3mut.

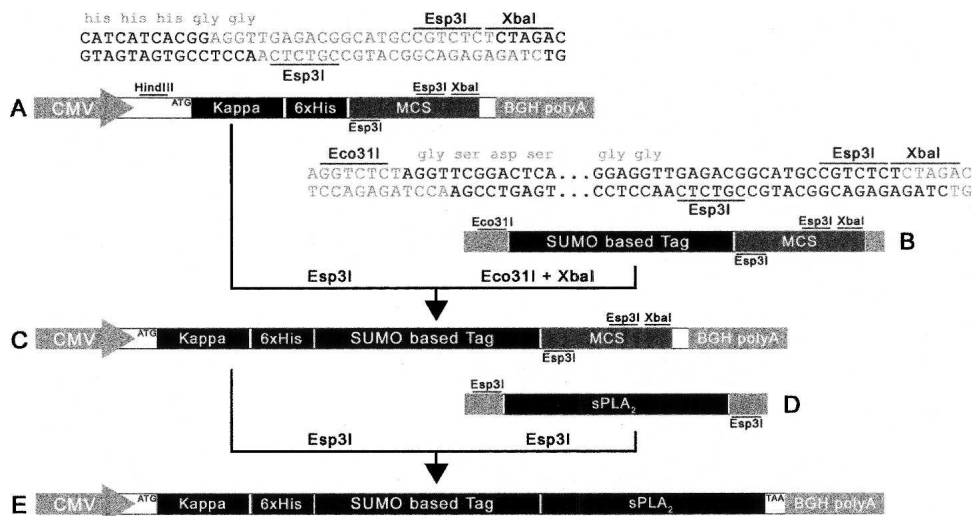


Figure 8. Representative detail of vector construction and sPLA₂ fusion. Initially, pcDNA3.1 was modified by inserting a kappa secretory signal followed by a 6xHis tag and a modified multiple cloning site via a HindIII/XbaI double digestion. The resulting vector (A) was used directly to create 6xHis-tagged sPLA₂ constructs. In order to generate the various SUMO fusions, vector A was digested with Esp3I and ligated to Eco31I/XbaI digested, PCR-amplified, SUMO derivatives containing a new multiple cloning site (B) to give vector C. SUMO vectors (C) were digested with Esp3I and fused with sPLA₂ PCR products (D) digested with Eco31I, Esp3I, or BpiI. This strategy resulted in the creation of in-frame fusions (E) between the various SUMOs and sPLA₂s.

Transient transfection in HEK-293T and CHO-K1 cells

HEK-293T or CHO-K1 cells were seeded into six-well plates (Becton Dickinson) at a density of 500,000 cells per well in either DMEM or F12, respectively, containing 10% fetal bovine serum media, and incubated overnight at 37°C with 95% air/CO₂. Cells were transiently transfected with various sPLA₂ constructs in pcDNA3.1 vector (2.5 µg/well) using Lipofectamine-LTX as described by the manufacturer (Invitrogen). After transfection, cells were incubated for an additional 48 h at 37°C before being analyzed for PLA₂ expression.

Expression analysis

After 48 h of incubation, following transfection, media and cells were collected for analysis. Culture media was removed from each well (~1.5 mL), and debris was separated by centrifugation. For SDS-PAGE/Western blotting, media was mixed with 6× SDS loading buffer and boiled for 5 min. The remaining media was stored at -80°C for later assay. Cells were washed from each well of the plate, separated by centrifugation, re-suspended in 180 µL of cold RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% Na deoxycholate), sonicated briefly, mixed with 6× SDS loading buffer, and boiled for 5 min. All samples were resolved on denaturing 15% acrylamide gels with a 4% acrylamide stacking layer. Gels were transferred to Immobilon nitrocellulose (Millipore) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). After transfer, blots were blocked with 5% non-fat milk in PBS (pH 7.5) + 0.05% Tween 20 (PBST) for 1 h. Following blocking, the blots were incubated in 1:1000 monoclonal Anti-His Antibody (Sigma) in PBST + milk for at least 1 h. Blots were washed with PBST three times and incubated with 1:2500 anti-mouse HRP conjugated antibody (Sigma) in PBST + milk for 1 h. Blots were again washed three times with PBST. HRP conjugates were detected with SuperSignal West Pico chemiluminescent substrate (Pierce). Blots were imaged using an LAS-3000 (Fujifilm Life Science). Densitometry analysis was performed using Multi Gauge 3.0 (Fujifilm Life Science). Density was calculated by subtracting background and dividing by area. All areas compared were of equal size, while exposures were set to ensure that none of the bands compared had reached saturation. Standard curves were created for quantification by fitting known sample values to the function $Y = aX^2 + bX + c$ in Multi Gauge. The software was then used to determine the relative amounts of the various SUMOstar fusions (loaded on the same gel). Quantities of the other constructs were determined by comparing density values and adjusting for differences relative to SUMOstar. For example: The average value of SUMOstar-PLA₂-IIE expression, found by a standard curve fit, was 4.98 ng/µL, and the ratio in densities between SUMO-sPLA₂-IIE and SUMOstar-sPLA₂-IIE expression in Figure 7A was 3:5, respectively; so SUMO-sPLA₂-IIE expression was calculated to be 2.98 ng/µL.

Purification of sPLA₂ group X

Small-scale purification was performed in batch mode using Ni²⁺-NTA beads (QIAGEN). Briefly, 50 µL of Ni²⁺-NTA beads was equilibrated with wash buffer (0.05 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.2 M imidazole). Clarified medium (1 mL) was adjusted to 0.02 M imidazole by addition of 1 M imidazole in 0.05 M Tris-HCl, pH 7.5, 0.5 M NaCl. Samples were gently mixed with beads for at least 1 h at 4°C, washed three times with wash buffer, and eluted with wash buffer containing 0.5 M imidazole.

Larger-scale purification was accomplished on a 5-mL HisTrap FF Ni²⁺ Sepharose column (GE Healthcare). Conditioned medium was filtered through a 0.2-µm filter, adjusted to 20 mM imidazole, and loaded onto the column at a flow rate of 1 mL/min. The column was washed with 95% Buffer A (0.05 M Tris-HCl, pH 8.0, 0.5 M NaCl) and 5% Buffer B (Buffer A + 0.5 M imidazole). Elution was performed in two steps: first with 90% A/10% B, and then with 100% B. Fractions were analyzed for sPLA₂ activity following digestion with SUMOstar protease (LifeSensors). Eluted fractions with sPLA₂ activity were pooled, concentrated, and exchanged into 0.05 M Tris-HCl pH 7.5 and 2 mM CaCl₂.

Assay

Enzymatic activity was analyzed according to the method of Nicholson (Nicholson et al. 2008). Briefly, SUMOstar-sPLA₂ was mixed with 20 nM SUMOstar protease and 20 µM NBD C6-HPC (Invitrogen). Release of NBD-fluorescence was measured at 1-min intervals in a FluorScan plate reader (ThermoScientific) using an excitation wavelength of 460 nm and emission wavelength of 538 nm. Following an initial lag period reflecting the activation of sPLA₂ through removal of the SUMOstar fusion partner, steady-state PLA₂ activity was determined.

Acknowledgments

We thank Dr. Michael Gelb for his generous gift of the group X sPLA₂ clone and Drs. James Strickler and Michael Mattern for helpful discussions and critical reading of the manuscript. Part of this work was funded by NIH/NIGMS grants, GM067271-02 and GM068404-3.

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