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Production of Sumoylated Proteins Using a Baculovirus Expression System

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Summary

Spodoptera frugiperda Sf9 cells were found to possess an active endogenous sumoylation system. However, the endogenous sumoylation machinery did not efficiently modify exogenous proteins expressed by infection with recombinant baculoviruses. To overcome this limitation, mammalian sumoylation components were introduced by co-infection with recombinant baculoviruses expressing individual protein components of the sumoylation pathway. Expression of mammalian Ubc9 plus SUMO (either SUMO1 or SUMO3) was necessary and sufficient for active sumoylation of co-infected test proteins. This system provides a simple and convenient means to produce sumoylated mammalian proteins in a eukaryotic environment. Large-scale cultures should provide quantities of sumoylated proteins sufficient for potential purification.

Keywords

Baculovirus; Wrn; Ubc9; SAE; SUMO; sumoylation

1. Introduction

The baculovirus expression vector system (BEVS) has been extensively used since its inception 23 years ago (Smith et al., 1983) to express a large variety of proteins. The most attractive feature offered by the BEVS is its ability to produce significant amounts of the desired protein in a cellular environment that provides the appropriate conditions to allow the correct folding, targeting, and post-translational modification of the expressed protein (King and Possee, 1992). Therefore, proteins produced by the BEVS are more likely to exhibit the full range of characteristics displayed by the original proteins under their natural host environment than proteins expressed in bacterial systems. Among the numerous protein properties found to be accurately recapitulated by the BEVS are tertiary structure, disulfide links, formation of homo and hetero-multimeric protein complexes, phosphorylation, membrane targeting and insertion, and cellular localization.

About 10 years ago a novel ubiquitin-like post-translational modification known as sumoylation was characterized (Mahajan et al., 1997; Matunis et al., 1996). Sumoylation

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Competing Interests Statement

The authors declare no competing interests associated to this report.

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involves the modification of the target protein by the covalent linkage of a SUMO (Small Ubiquitin-like Modifier) molecule via an isopeptide link between the carboxyl-group in the C-terminal glycine in SUMO and the epsilon amino group in a lysine residue located in the target protein. Sumoylation involves a series of sequential enzymatic steps mediated by a set of enzymes exclusive to this pathway (Figure 1). The enzymatic components of the pathway appear to be present in all eukaryotic organisms and exhibit substantial functional and structural conservation throughout the evolutionary scale. This is best exemplified by the observation that the human SUMO conjugating enzyme hUbc9 can complement a *Saccharomyces cerevisiae* mutant exhibiting a temperature sensitive mutant of the yeast counterpart for the SUMO conjugating enzyme Ubc9 (Yasugi and Howley, 1996). The most divergent components of the sumoylation system are the modifiers themselves, which vary significantly in number according to the genome size of the organism: 8 different SUMO modifiers have been described in *Arabidopsis thaliana* (Kurepa et al., 2003), whereas 4 have been described in mammals and only one is present in fission and budding yeasts [reviewed by Larsen and Köhler (Larsen and Köhler, 2004)]. The human SUMO2 and SUMO3 are very closely related and appear functionally divergent from SUMO1 as indicated by the limited overlap observed in the set of cellular substrates modified by SUMO2/3 and SUMO1 (Rosas-Acosta et al., 2005).

Sumoylation exerts a large variety of effects on its targets, including alterations in their enzymatic activity, cellular localization, ability to interact with other proteins, and stability [reviewed by Johnson (Johnson, 2004) and Dohmen (Dohmen, 2004)]. For most known SUMO substrates, the fraction of protein that is sumoylated *in vivo* represents only a very small share, usually less than 5%, of the total cellular amount of the protein. This has made it difficult to characterize the mechanisms that mediate the effects exerted by sumoylation on protein function. To overcome the low yield of sumoylated proteins in vertebrate cell culture, two groups developed similar methodologies to allow the expression and purification of sumoylated proteins in bacteria (Mencia and de Lorenzo, 2004; Uchimura et al., 2004). While these bacterial system can produce biochemically useful quantities of some sumoylated proteins, many eukaryotic proteins express poorly in prokaryotic cells due to codon bias or solubility issues. In addition, bacterial sumoylation systems cannot be used to explore the relationship between sumoylation and other eukaryotic-specific post-translational modification, or the effect of sumoylation on intracellular localization. As an alternative to the bacterial systems, the use of the BEVS was explored as a way to produce sumoylated proteins in a eukaryotic environment. To this end, recombinant baculoviruses were developed for each component of the SUMO pathway and observed the effect mediated by single and multiple co-infections on total cellular sumoylation and on the sumoylation of well characterized exogenous SUMO substrates. The results indicated that over-expression of SUMO and the conjugating enzyme Ubc9 are required and sufficient both to induce a significant increase in total cellular sumoylation and to stimulate the sumoylation of an exogenous protein expressed by co-infection. Use of this system should allow the production of sumoylated proteins that closely match all the functional properties of the original protein.

2. Materials and methods

2.1. Materials, cells and viruses

Spodoptera frugiperda Sf9 cells, the baculovirus transfer vectors pBACgus-1 and pBACgus-2cp, the BacVector-3000 triple cut *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcMNPV) DNA, the Eufectin transfection reagent, and the HRP-conjugated S protein were all from Novagen. TNM-FH medium and Fetal Bovine Serum were from HyClone. The wild type AcMNPV-E2 virus was a gift from Dr. Max D. Summers (Dept. of Entomology, Texas A&M University, College Station, TX).

2.2. Tissue culture and development of recombinant viruses

Sf9 cells were maintained in suspension at a density of $0.5 - 3.0 \times 10^6$ cells/ml in TNM-FH medium supplemented with 10% v/v FBS and 0.1% w/v Pluronic F68. The growth temperature was maintained at 27°C throughout all the experiments described. To develop the constructs required for the expression of the different components of the sumoylation pathway in the BEVS, the ORFs for the human genes SUMO1, SUMO3, SAE1, SAE2, and Ubc9, and the rat gene PIASx β (Miz1) were each individually cloned into pBACgus baculovirus transfer vectors. The pBACgus2cp vector, encoding for an N-terminal His-tag followed by an S-peptide tag, was used for SUMO1 and SUMO3. The pBACgus1 vector, which does not confer any tag or exogenous sequence to the cloned gene, was used for all other genes. The resulting recombinant transfer vectors were sequenced to confirm the reading frame and sequence of the corresponding gene inserted. To develop recombinant viruses, 2×10^6 Sf9 cells were plated in T25 flasks and were co-transfected with 500 ng of the appropriate recombinant transfer vector DNA and 100 ng of BacVector-3000 triple cut AcMNPV DNA, using Eufectin as directed by the manufacturer. Culture supernatants were collected 5 to 7 days post-transfection and the recombinant baculoviruses produced were isolated by plaque assay. The isolated recombinant viruses were amplified to generate high titer viral stocks according to standard techniques. The presence of the desired exogenous gene in each recombinant baculovirus produced was confirmed by PCR amplification using primers specific to the exogenous gene and by immunoblotting analyses using antibodies directed against the exogenous protein expressed.

2.3. Viral infections, SDS-PAGE and immunoblot analyses of infected cells

Sf9 cells were seeded in T25 flasks at a density of 2.0×10^6 per flask and allowed to adhere to the flask for at least 1 hour. Then, high titer viral stocks were added to obtain the desired multiplicity of infection (MOI). When two or more different viruses were co-infected, each viral stock was added as above. Upon infection, the cells were maintained for the desired time at 27°C. The infected cells were collected by sloughing, counted, washed with ice cold 1x PBS, spun down, and resuspended at a final density of 2×10^6 cells/ml in a 1:1 mix of 1x PBS and 4x Sample Buffer (100 mM Tris pH 6.8, 20% Glycerol, 8% SDS, 0.02% Bromophenol Blue, and 4% β -Mercaptoethanol) for direct immunoblotting or **processed for immunoprecipitation as previously described (Rosas-Acosta et al., 2005)**. The appropriate volumes for the indicated number of cells per well were then loaded on a 10% SDS-PAGE gel, run, and transferred to Immobilon-PTM membranes at 1.4 Amps for 90 minutes. The membranes were blocked with 3% non-fat milk for 30 minutes at room temperature, and incubated overnight at 4°C with the indicated primary antibody or with S-Protein HRP conjugate at a 1:5,000 dilution. The antibodies used were anti-SUMO (rabbit serum #12783), anti-SAE2 (rabbit serum #12739), anti-Ubc9 (rabbit serum #12741), anti-XpressTM monoclonal antibody R910-25 (Invitrogen Corp., Carlsbad, CA), anti- α -Tubulin monoclonal antibody sc-5286 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-SAE1 sheep serum ALX#210-328-C050 (Alexis Corporation, Lausen, Switzerland), and anti-6x-His tag monoclonal antibody #631212 (BD Biosciences, Palo Alto, CA). All of the above rabbit sera (#12783, #12739, and #12741) were produced in house using the corresponding purified human proteins as immunogens, as previously reported for the SUMO serum (Rosas-Acosta et al., 2005). All secondary antibodies used were HRP conjugated antibodies from Santa Cruz Inc., and were always used at a 1:5,000 dilution. Immunoblots were developed by chemiluminescence using the Western LightningTM chemiluminescence reagent (Perkin Elmer Life Sciences, Inc., Boston, MA).

3. Results and discussion

3.1. Endogenous sumoylation in Sf9 cells

Production and purification of specific sumoylated proteins from mammalian cells is difficult due to the relatively low abundance of the sumoylated forms that are typically present. As an

alternative to mammalian cell culture, the use of the highly efficient baculovirus expression vector system (BEVS) was explored as means to produce larger quantities of sumoylated proteins. The sumoylation system is present and active from yeast to mammals (Johnson, 2004), including insects as exemplified by *Drosophila melanogaster* (Lehembre et al., 2000; Long and Griffith, 2000; Shih et al., 2002). Sumoylation has also been reported in the *Spodoptera frugipuda*-derived cell line, Sf9, which is typically used for the BEVS (Leight et al., 2005), though this modification process has not been studied in detail in these cells. To determine the extent of endogenous sumoylation in Sf9 cells, cell extracts were prepared from uninfected cultures and analyzed by immunoblotting with a previously described rabbit anti-SUMO1 serum (Rosas-Acosta et al., 2005). Both free SUMO and higher molecular weight species were observed, indicating the presence in Sf9 cells of both an endogenous SUMO exhibiting significant cross-reactivity with human SUMO1, and an active sumoylation process (Fig 2A, lane 1). To determine if baculovirus infection exerted a stimulatory or inhibitory effect on the host cell's sumoylation system, Sf9 cells were infected with wild-type *Autographa californica* Multiple Nuclear Polyhedrosis virus strain E2 (AcMNPV-E2). Following infection there was a decrease in the amount of free SUMO coupled with an increase in the amount of SUMO-conjugated products, consistent with a baculovirus-induced stimulatory effect on sumoylation that likely reflects a general host cell response to infection. However, after infection with several different baculoviruses encoding various mammalian proteins, there was little or no sumoylation of the exogenously expressed products (data not shown and see Fig. 5).

3.2. Baculovirus-mediated expression of functional mammalian sumoylation components in Sf9 cells

In mammalian cells, effective sumoylation of exogenously expressed proteins typically requires overexpression of at least some of the sumoylation system components. Likewise, it was postulated that co-expression of the mammalian SUMO components with the target transgene would enhance sumoylation of foreign proteins in Sf9 cells. Therefore, baculovirus expression vectors for mammalian SUMO1, SUMO3, Ubc9, SAE1, and SAE2 were constructed to test their effect on transgene sumoylation. Recombinant SUMO1 and SUMO3 baculoviruses were constructed with N-terminal His- and S-tags to distinguish them from the endogenous Sf9 SUMOs and to facilitate affinity purification of sumoylated products. In initial studies, infections with each construct were performed at MOIs of 1, 2, 5, and 10, and expression was monitored by immunoblotting at 24 hr intervals for 5 days. For each construct, an MOI of 5 was determined to produce optimal yield of protein (data not shown). Fig. 3 shows the time course of expression of five cloned SUMO components with infections done at an MOI of 5. All of these proteins, except SAE2, were well expressed and accumulated to significant amounts by 48 hrs post-infection. SAE2 was detectable, but to a much lower extent. Whether the poor detection of SAE2 was due to actual low protein levels or to ineffective reactivity with the anti-SAE2 antibody has not been resolved.

While all of the mammalian sumoylation components could be expressed in Sf9 cells, individual expression of SUMO1 or SUMO3 resulted in very little conjugation of either exogenous SUMO to cellular and viral proteins (data not shown and Fig. 4), implying that the mammalian SUMOs were not efficiently utilized by the insect sumoylation enzymes. Consequently, co-infection experiments were performed using various MOI ratios of the SUMO-expressing viruses (either SUMO1 or SUMO3) plus the 3 other viruses expressing mammalian Ubc9, SAE1, and SAE2, respectively. Total conjugation of S-tagged SUMO into host cell and/or viral proteins was monitored by immunoblotting with HRP-conjugated S protein. At all MOI ratios tested there was detectable conjugation with the exogenous SUMOs, indicating that the tagged, mammalian SUMOs were functionally active in Sf9 cells when supplied with the cognate sumoylation enzymes (data not shown and Fig. 4).

During the MOI ratio experiments, it was observed that varying the amount of SAE1/2 in the co-infections had minimal effects on sumoylation efficiency. These results led us to investigate the specific requirements for reconstituting a functional mammalian sumoylation system in Sf9 cells. A series of co-infections with varying combinations of the sumoylation component viruses was performed, and sumoylation was detected by immunoblotting with the tag-specific antibody (Fig. 4A). As discussed above, infection of either SUMO1 or SUMO3 in the absence of the mammalian sumoylation enzymes resulted in good SUMO expression, but little conjugation (lanes 2 and 9). Triple co-infection of the SUMO1 or SUMO3 viruses with both the SAE1 and SAE2 viruses did not enhance sumoylation (lanes 3 and 10); however, co-infection of SUMO with just Ubc9 (lanes 6 and 13) stimulated conjugation to the same extent as the quadruple co-infection with SUMO plus Ubc9, SAE1, and SAE2 (lanes 4 and 11). The conclusion from these results is that the insect SAE1/2 enzyme was sufficient for activation of the mammalian SUMOs, and that there was no specific requirement in this reconstitution system for exogenous mammalian SAE1/2. Furthermore, these results imply that the mammalian Ubc9 and SUMOs constitute the limiting factors for efficient sumoylation, though the basis for this requirement has not been explored. Lastly, it was observed that sumoylation in this *in vivo* reconstitution system could be even further enhanced by additional co-infection with recombinant baculovirus expressing SUMO ligase, Miz1 (PIASx β), further supporting that this baculovirus expression system faithfully recapitulates the biochemistry of sumoylation (lanes 5, 7, 12, and 14).

3.3. Sumoylation of exogenous proteins in Sf9 cells with a reconstituted mammalian sumoylation system

After establishing the conditions for expression of the mammalian sumoylation system in Sf9 cells, the reconstituted system was tested for its ability to sumoylate exogenous mammalian proteins. Shown are the results for the Werner's helicase protein, Wrn (Fig. 5). Wrn is known to be an efficient sumoylation target *in vivo* and is multiply sumoylated (Kawabe et al., 2000). Infection of Sf9 cells by a baculovirus vector expressing wildtype Wrn resulted in excellent expression of full-length Wrn (Fig. 5A, lane 2). However, as for the other previously tested exogenous proteins, there was no detectable modification of exogenous Wrn by the endogenous Sf9 sumoylation system under these conditions. In contrast, co-infection of the Wrn vector with the Ubc9 plus SUMO1 viruses resulted in the appearance of multiple, higher molecular weight species of Wrn (Fig. 5A, lane 3), consistent with the previously observed sumoylation pattern for this protein (Kawabe et al., 2000; Woods et al., 2004). **To confirm that these higher molecular weight forms were truly sumoylated Wrn products, Wrn was immunoprecipitated, and the precipitates were analyzed by immunoblotting** (Fig. 5B). First, the blot was probed at low sensitivity with an anti-Express tag antibody to detect Wrn (left panel). The blot was then reprobbed with anti-SUMO (right panel) and revealed SUMO-specific forms that were present only in the Wrn immunoprecipitates from samples expressing the sumoylation components (lanes 7 and 8), confirming that the bands contained both Wrn and SUMO. The ability of Wrn to be sumoylated upon co-infection with Ubc9 and SUMO1 baculoviruses confirms the successful reconstitution of mammalian sumoylation in Sf9 cells. Similar sumoylation results were obtained for p53 and bovine papillomavirus E1 protein (data not shown), suggesting that this methodology is generally applicable.

The study presented here demonstrates that transient expression of mammalian sumoylation components by co-infection with recombinant baculoviruses in Sf9 cells results in functional sumoylation of exogenous proteins that are not sumoylated by the endogenous insect sumoylation machinery. Only two mammalian components were required for reconstituting functional sumoylation, the conjugation enzyme (Ubc9) and a SUMO protein. Exogenous expression of the heterodimeric mammalian activating enzyme (SAE1/2) was not required, indicating that the Sf9 SAE1/2 equivalent must function adequately with mammalian SUMO

and Ubc9. Using this simple co-infection system, exogenous proteins could be expressed and sumoylated. Since the exogenous SUMO construct used in these studies has a tandem-affinity tag incorporated, this could provide a convenient mechanism for specific purification of the sumoylated species such as previously demonstrated for a SUMO-expressing cell line (Rosas-Acosta et al., 2005).

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Figure 1.

The SUMO modification system. SUMO conjugation involves a series of sequential enzymatic steps, namely proteolytic processing of the SUMO precursor by the Ubiquitin-like specific proteases (ULPs) to expose the internal di-glycine motif in SUMO, ATP-dependent activation by the E1 heterodimeric enzyme SAE2/SAE1, and conjugation of SUMO to the target protein mediated by the E2 conjugating enzyme Ubc9. The conjugating step is enhanced by E3 ligases. Upon conjugation, SUMO can be subsequently removed from the target by the isopeptidase activity of the ULPs. Expression of a truncated SUMO (ending in the di-glycine motif) avoids the need for proteolytic processing.

Figure 2.

Baculovirus infection stimulates sumoylation in Sf9 cells. Uninfected Sf9 cells (UI) or Sf9 cells infected with *Autographa californica* Multiple Nuclear Polyhedrosis Virus AcMNPV-E2 wild type (WT) baculovirus at MOI=5 were analyzed by SDS-PAGE and immunoblotting using rabbit serum raised against human SUMO1. Each lane corresponds to 1.0×10^5 cells. A significant decrease in unconjugated endogenous SUMO (arrow), accompanied by a significant increase in the amount of conjugated endogenous SUMO (bracket) was observed upon baculovirus infection. The position of molecular weight markers is indicated on the left.

Figure 3.

Time course of expression of the different sumoylation system components in Sf9 cells infected with single recombinant baculoviruses. Spin flasks containing Sf9 cells at a density of 2×10^6 cells/ml were infected at MOI=5 with a single recombinant baculovirus expressing the indicated protein. Samples (5 ml) were taken every 12 h post-infection (h p.i.), counted, and a volume equivalent to 4×10^4 cells was analyzed by SDS-PAGE (10% gel) and immunoblotting. SUMO1 and SUMO3 were detected using HRP-conjugated S-Protein. Ubc9 and SAE2 were detected using rabbit serum raised against the corresponding human enzymes. SAE1 was detected using a commercially available sheep serum.

Figure 4.

Total sumoylation in Sf9 cells co-infected with different combinations of recombinant baculoviruses expressing sumoylation system components. T25 flasks containing 2.0×10^6 Sf9 cells were infected with different combinations of recombinant baculoviruses expressing SUMO1 or SUMO3 (MOI = 5), SAE1 (MOI = 2), SAE2 (MOI = 2), Ubc9 (MOI = 5), and Miz1 (MOI = 2). At 72 h post-infection, the cells were collected, counted, and a volume equivalent to 4×10^4 Sf9 cells was analyzed by SDS-PAGE (10% gel) and immunoblotting, using either monoclonal antibodies against His-tag (A) or α -Tubulin (B), sheep serum for SAE1 (C), or rabbit serum specific for Ubc9 (D). The star indicates conjugated forms of SUMO and the empty arrow indicates unconjugated SUMO. The positions of molecular weight markers are indicated on the left in kDa.

Figure 5.

In vivo sumoylation of an exogenous target expressed in Sf9 cells. (A) T25 flasks containing 2×10^6 cells were infected with different combinations of recombinant baculoviruses expressing SUMO1 (MOI = 5), Ubc9 (MOI = 5), and Wrn (MOI=10). At 72 h post-infection, the cells were collected, counted, and a volume equivalent to 4×10^4 cells was analyzed by SDS-PAGE (5% gel) and immunoblotting using a monoclonal antibody against the Xpress™ epitope (DLYDDDDK), located at the N-terminus of Wrn. Unmodified (non-sumoylated) Wrn is indicated by the empty arrow. Sumoylated forms of Wrn are indicated by the filled arrows. Molecular weight markers are indicated on the left in kDa. **(B) Flasks were infected as in (A) using recombinant baculoviruses expressing the following: SUMO1, Ubc9, and Miz1 (lanes 1); Wrn (lanes 2); Wrn, SUMO1, and Ubc9 (lanes 3); and Wrn, SUMO1, Ubc9, and Miz1 (lanes 4). Cultures were harvested at 72 h post-infection and extracts prepared for immunoprecipitation of Wrn using an anti-Express tag antibody. The immunoprecipitates were resolved on a 5% polyacrylamide gel and immunoblotted with anti-Express (left panel) or anti-SUMO (right panel). The * indicates the unmodified Wrn, and the arrows indicate sumoylated forms of Wrn.**