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Suppressor

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13. ABSTRACT (Maximum 200 Words) Our investigation into the relationships between the host growth and transformation suppressor, PML and HCMV kinase IE72, has furthered our understanding of PML and its role in nuclear structure (POD) maintenance. PML/PODs are involved in cell cycle control, apoptosis, and oncogenesis. Therefore, we have used the viral kinase, IE72 as a tool to decipher the mechanisms of POD assembly and PML suppressor function. We have accumulated much data to support our hypothesis: We demonstrate that IE72 preferentially binds the SUMO-1 modified, POD-associated PML and phosphorylates PML, thereby mediating PML de-sumoylation and contributing to POD disruption. We also provide evidence that IE72 kinase activity regulates PML stability. We have narrowed the identification of the serine(s) involved in regulating PML stability to 9. It is our contention that the identification of these important regulatory phospho-amino acids will allow for the design of a PML based anti-cancer strategy.				
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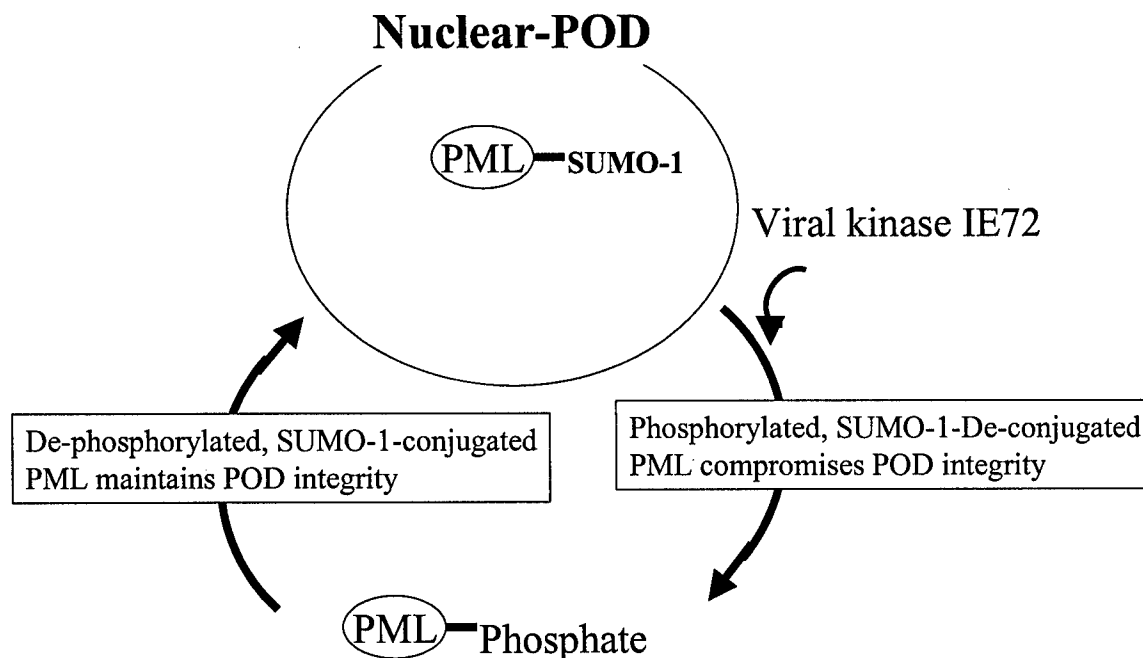
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Introduction

The objective of this investigation is to study the integrated relationships between the host growth and transformation suppressor, Promyelocytic Leukemia Protein (PML) and the Human Cytomegalovirus kinase (HCMV IE72) in order to facilitate the design of PML-based anti-cancer therapy. PML suppresses growth and tumorigenicity of human breast cancer cells by inducing G1 cell cycle arrest, apoptosis, and Class I antigen expression (1,2). Upon infection, IE72 immediately targets PML, which is associated with nuclear structures referred to as PML Oncogenic Domains (PODs), resulting in POD dispersal (3&4). PML dispersal is associated with increased levels of highly phosphorylated, mitotic PML (5) and loss of cell growth control, as well as inhibition of apoptosis, and gene expression. Therefore, IE72 exerts control over PML ostensibly at odds with PML growth suppressor function. Thus IE72 affords us with a valuable tool to use in the deciphering the regulation of PML tumor suppressor function. We have previously described a unique kinase function of IE72 (6) and now show that SUMO-1 modified PML is an IE72 substrate. Further, we demonstrate through the use of active and inactive-kinase IE72 that maintenance of POD integrity is regulated by reversible postranslational modifications (phosphorylation and sumoylation) of PML. Further, we demonstrate that a PML mutant, in 9 serine residues, is resistant to IE72 mediated degradation. PML is a potential candidate for the design of novel therapeutic approaches to the treatment of breast cancer, thus it is imperative that its mechanisms of action are understood and IE72 has proven to be a valuable tool in this endeavor.

BODY

We have accumulated much data to support our hypothesis, which is diagrammed and concisely stated below:



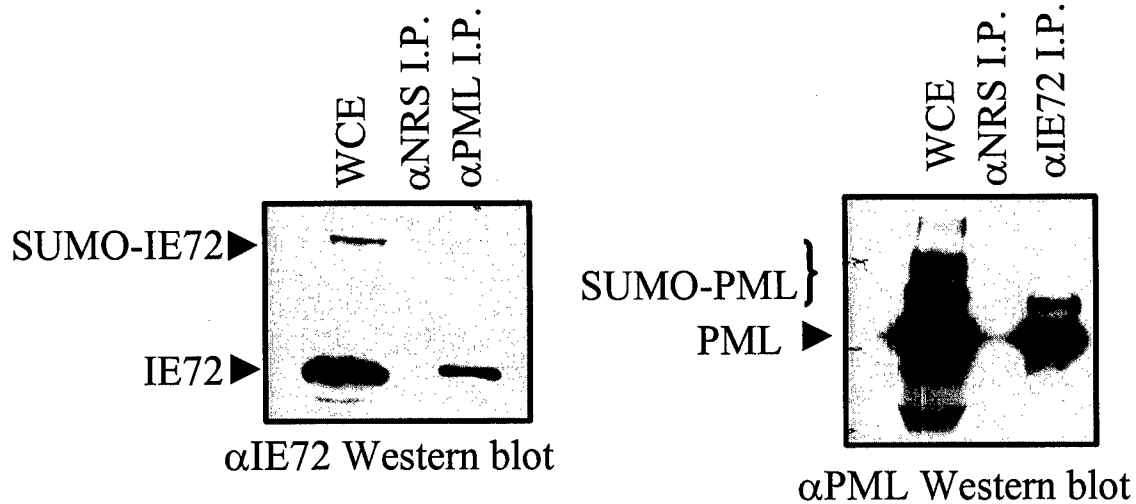
Hypothesis: IE72 phosphorylates SUMO-1 modified PML and subsequently mediates the desumoylation of PML and loss of POD integrity

IE72 preferentially binds to SUMO-1 modified PML, POD associated PML

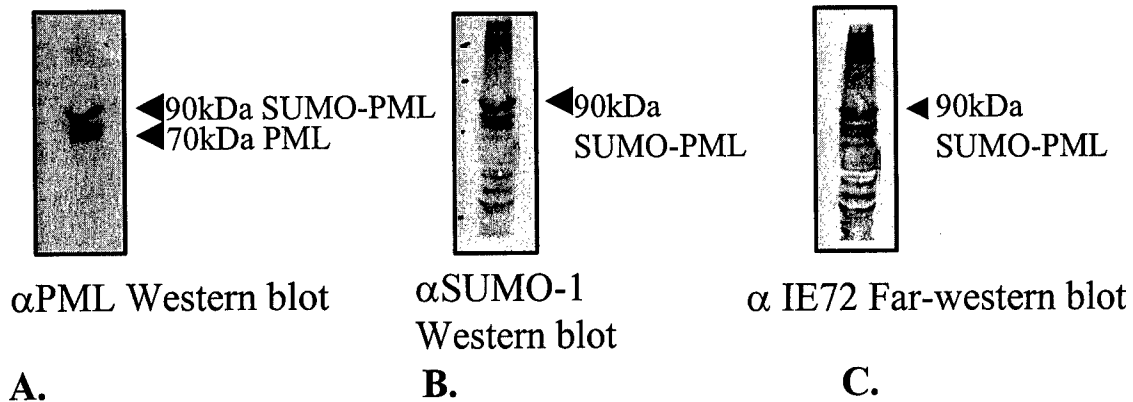
- 1.) To begin our investigation into the relationship between PML and IE72 we first asked if there was a direct physical interaction between these proteins. IE72 may target PODs through an affinity for PML. However, our initial attempts to demonstrate this interaction using coimmunoprecipitation and affinity pull down assays were not convincing. We then hypothesized that IE72 may have preferential affinity for SUMO-1-PML the less abundant POD associated form of PML. We increased the cell lysate concentrations of SUMO-1 modified PML by pretreating cells with arsenic and adding the specific sumo-1 hydrolase inhibitor (NEM) to cell lysates (7). The resulting coimmunoprecipitation experiments **demonstrated that these two proteins formed complexes in the cell and suggested that SUMO-1-modification of PML was important for this interaction.**

For some experiments, such as this one we opted not to use the SKBr cells because either they were resistant to viral infected particularly HCMV infection or because they contained low endogenous levels of PML. Western blot signals and Immuno-staining

with anti-PML resulted in smaller and less intense POD staining signals in SKBr compared to HHF and U373.

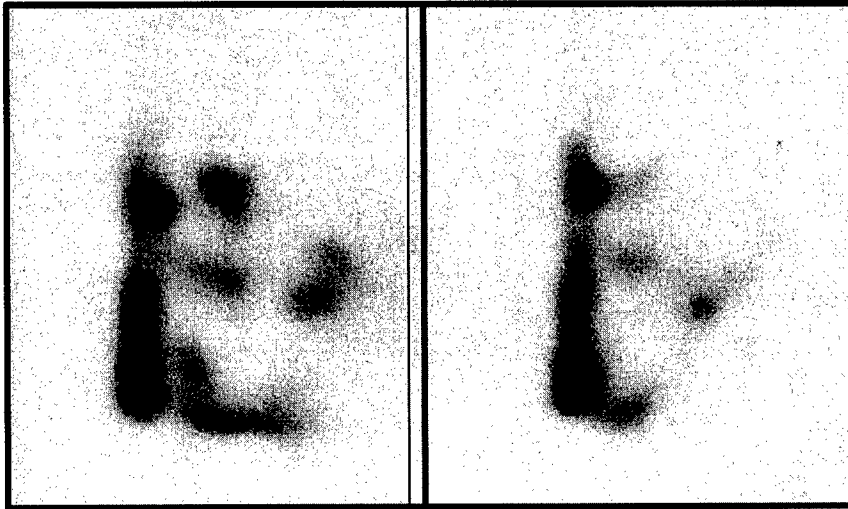


- 2.) To further substantiate that IE72 specifically targets the SUMO-1 modified forms of PML, a Far Western blot assay was performed. Cells were transfected with a plasmid expressing His-tagged PML and lysed directly in 6M Guanidine to prevent loss of the SUMO-1-modified PML. This lysate was bound to nickel resin, washed, resolved via SDS-PAGE, transferred to PVDF membrane and the proteins were renatured overnight. The membrane was blocked and incubated with either labeled IE72 (data not shown-The labeled IE72 did bind to SUMO-1 PML however the background was high) or purified GST-IE72. The bound GST-IE72 was visualized by immuno-staining with a monoclonal antibody directed against IE72 and the same blot was reprobed with anti-PML as well as anti-SUMO-1 antibodies. The anti-PML Western blot analysis showed both the 70kDa unmodified PML and the 90kDa SUMO-1 modified PML as reported previously (8). The anti-SUMO-1 Western blot confirmed the migration of the SUMO-1 modified PML at 90kDa, which was super-imposable on the 90kDa protein of the Farwestern blot. These data demonstrated that IE72 has specific affinity for the less predominant SUMO-1 modified form of PML.



We have recently obtained a PML mutant, which is sumoylation deficient and will use this PML protein as a negative control in a similar Farwestern assays as described above. The main difficulty with this assay is obtaining enough SUMO-1-modified PML for IE72 to bind in order to get a strong Farwestern blot signal.

IE72 phosphorylates PML *in vivo* and *in-vitro*

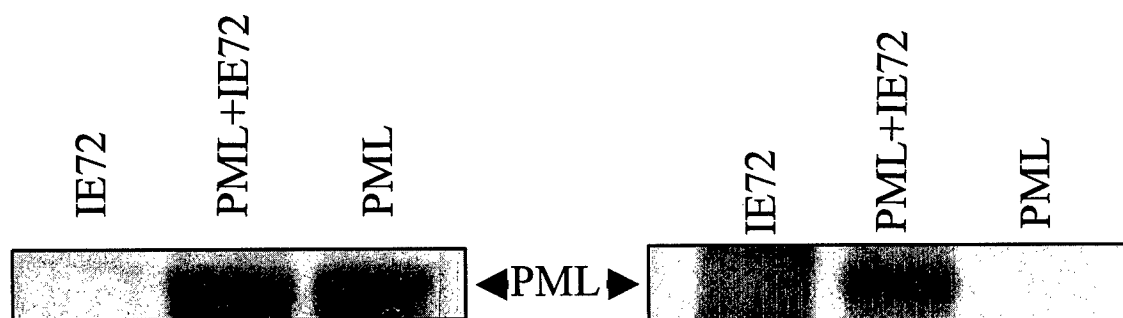


A. PML+IE72 B. PML+B-gal

1. To demonstrate the *in vivo* phosphorylation of PML in the presence of IE72, we infected cells with recombinant adenoviruses expressing PML and IE72. The cells were metabolically labeled with ortho phosphate and the cleared cell lysates were immunoprecipitated for PML. The I.P.s were SDS-PAGE resolved, transferred to PVDF membrane and the PML was excised. PML was digested with trypsin and the peptides resolved 2-dimensionally by electrophoresis and TLC. The resulting autoradiogram shows that cellular PML is more phosphorylated in the presence of IE72 compared to PML with out IE72.

Our initial attempts at this assay failed because we did not add NEM to the lysate. Also since IE72 phosphorylation of PML is an early event upon introduction of IE72 to the cell, we collected lysates within 7 hours of AD-IE72 infection.

2. To demonstrate the *in vitro* phosphorylation of PML by IE72, purified sepharose bound GST-PML was incubated with purified His-tagged IE72 and P^{32} ATP. Only PML incubated in the presence of IE72 was phosphorylated.



A. Protein stained gel

B. Autoradiogram

The main difficulties encountered in this assay were 1. The purification assay for IE72 active-kinase. We have learned that the buffer pH used in this purification is critical. 2. The purification of GST-PML is difficult. It is an unstable and insoluble protein. It helps to IPTG induce at low temperatures and maintain the protein in a pH of 8.

To facilitate our understanding of IE72 kinase-activity in POD disruption, we generated two kinase-deficient mutants: 1. a deletion in the DNA binding domain between aa 174-196 (IE72 Δ ATP) 2. a point mutant at leucine 174, which is substituted with proline (IE72P.M.174). Immuno-staining analysis was performed on POD structure as it is seen in the presence of an IE72 active and inactive-kinase.

SKBr were transfected either with pSG-IE72, pSG-IE72 Δ ATP, or pSG-IE72 P.M.174. 48 hours post transfection, cells were fixed, and co-immunostained with anti-IE72 and anti-PML antibodies. Secondary antibodies were tagged with Rhodamine (right panels-IE72 transfected cells) and FITC (left panels-PML staining). In cells that do not express IE72, PML is localized within sub-nuclear structures referred to as Promyelocytic Oncogenic Domains (PODs), whereas cells transfected expressing IE72 exhibit POD disruption. Kinase-deficient IE72 is not capable of disrupting PODs.

Kinase-active IE72 disrupts PODs

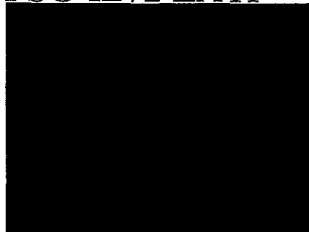
SkBr transfected with pPSG-IE72



SkBr transfected with pPSG-IE72 P.M. 174



SkBr transfected with pPSG-IE72 Δ ATP

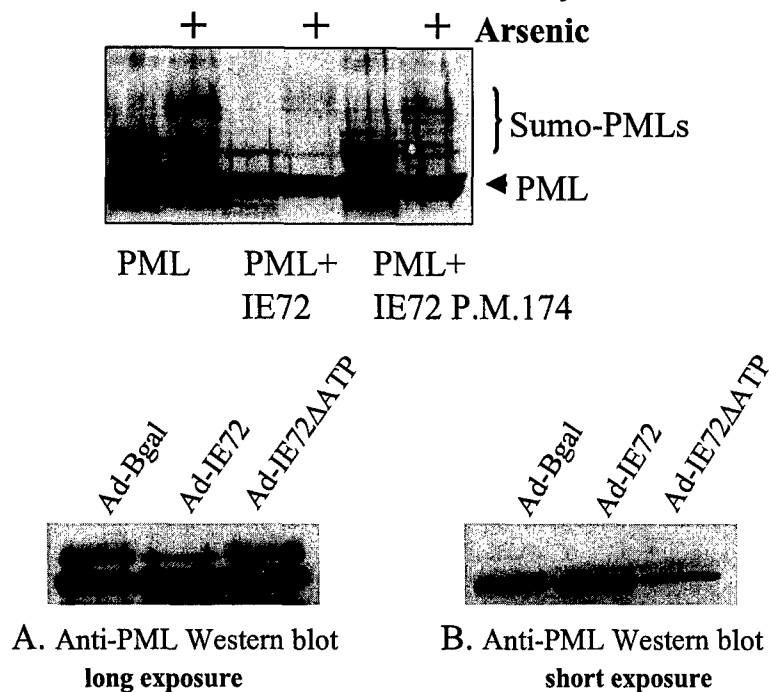


anti-PML

anti-IE72

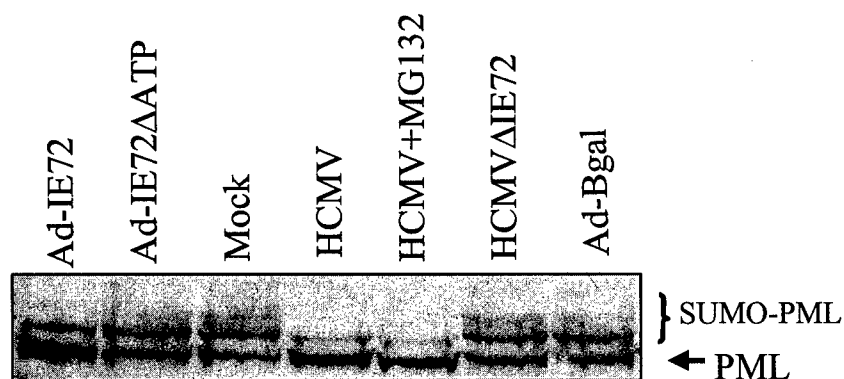
Our hypothesis states that IE72 mimics cellular kinase(s) which are active during mitosis, at which time a highly phosphorylated and de-sumoylated PML emerges. (5). Although tryptic peptide maps demonstrate that PML is differentially phosphorylated in the presence of IE72 this phosphorylated PML is not easily distinguishable upon analysis of Western blots. However these protein SDS-PAGE migration differences are more visible when comparing Western blot profiles of truncated PML +/- the presence of IE72 (data not shown). It is clear that IE72 mediates the loss of the SUMO-1 modified forms of PML and upon closer examination of the protein profiles we see that there is a concomitant increase in the unconjugated PML as the SUMO-1-conjugated forms of PML disappear. A similar observation has been reported in the emergence of "mitotic" PML. We interpreted this observation to suggest that IE72 mediates the loss of SUMO-1 modified PML through the process of de-sumoylation and not through ubiquitination/degradation as has been reported in the case of IE72 functional homologue, HSV ICPO. Since phosphorylation negatively regulates PML sumoylation (12), we hypothesize that IE72 phosphorylation of PML facilitates the desumoylation of PML. **The summation of these experiments supports the hypothesis that IE72 mimics cellular kinases creating a highly phosphorylated and de-sumoylated PML.**

Kinase-active IE72 mediates the de-sumoylation of PML



It has been reported that HSV ICPO and HCMV IE72 mediate the loss of SUMO-1-PML (10). The mechanism by which HSV ICPO induces this loss is via the ubiquitination/proteosome pathway (11). However we hypothesize that the mechanism used by IE72 to mediate the loss of SUMO-1 PML is different in that it involves IE72 phosphorylation of PML which then facilitates the de-sumoylation of PML (12). We followed the methods used to demonstrate that HSV mediated loss of SUMO-1 modified PML through the proteosome degradation pathway by using the proteosome inhibitor, MG132 (11). The results demonstrate that MG132 did not prevent HCMV-mediated loss of SUMO-1 modified PML. **These results lend further credence to the IE72 mediated de-sumoylation of PML. In the same Western blot, we show that IE72 alone is responsible for the de-sumoylation of PML since a HCMV deficient in IE72 expression has no effect on PML protein profile compared to mock infected cells.**

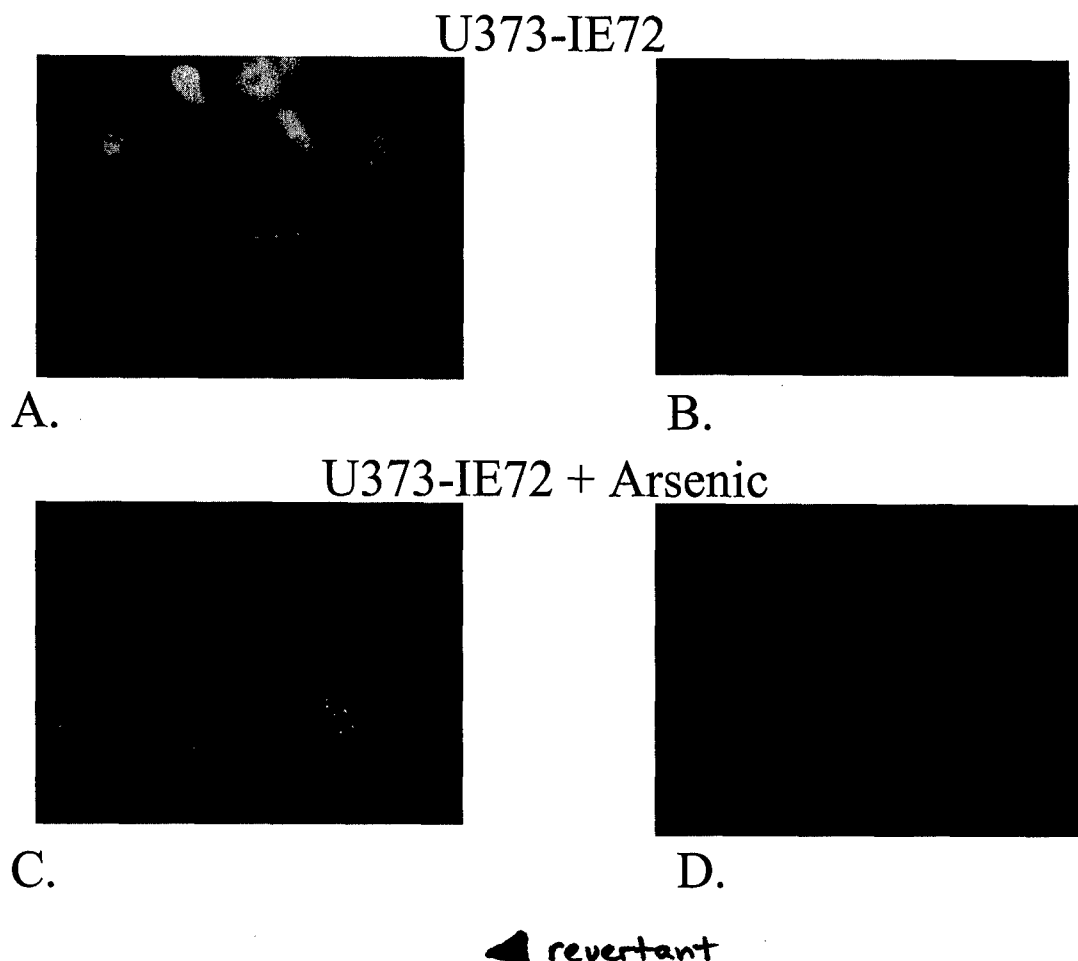
IE72 alone is responsible for PML de-sumoylation



As seen in this Western blot, it is difficult to detect the endogenous SUMO-1 modified forms of PML. We had more success when we used Dr. Roel Van Driels monoclonal antibody compared to commercially purchased PML antibody (13).

According to our model, PML of lower phosphorylated state and SUMO-1 conjugated facilitates PML aggregation into PODs. To further test this hypothesis, arsenic was added to cells stably expressing IE72. Arsenic is believed to decrease phosphorylation and increase sumoylation of PML. Therefore we wondered if arsenic could over-ride IE72 phosphorylating and desumoylating affect on PML and allow the reformation of PODs. Interestingly arsenic did allow the reaggregation of PML, however the structures formed with in an hour of arsenic treatment are not true mature PODs since they are more numerous and smaller. This would suggest that arsenic may not fully over-come IE72 affects on POD structure or that other variables may be involved.

U373 stably expressing IE72 form primary POD-like structures with arsenic treatment



We attempted to determine the PML amino-acid(s) phosphorylated by IE72.

We conclude that this residue(s) does not lie within the first 48 amino acids nor after amino acid 555 since these PML truncates can form POD structures, which are disrupted by IE72. We believe that the PML residue phosphorylated by IE72 may lie within aa 225-588 since a GST fusion of this mutant PML can be *in vitro* phosphorylated by IE72.

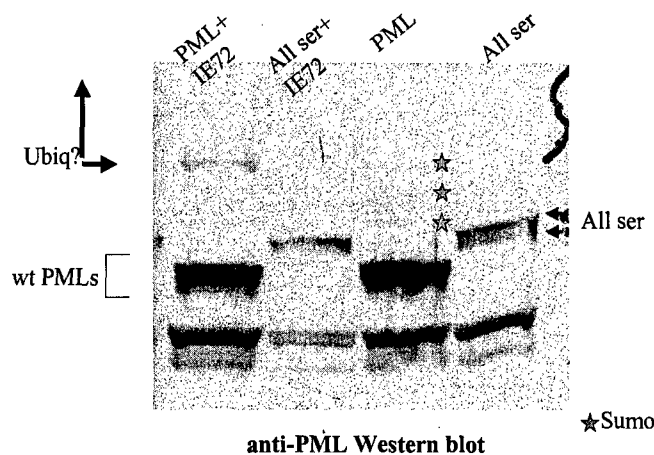
From the peptide mapping results, we isolated the PML tryptic peptide fragment with the strongest phosphorylation signal, and subjected it to mass spectrometry analysis. **The summary report has been added to the appendix.** The results revealed that IE72 mediated the phosphorylation of a PML serine residue, which was preceded by a proline. Amino acid analysis of PML reveals 9 such serines exist within PML. This PML mutant had already been constructed by Dr. Kun-Sung Chang (Anderson Cancer Center, Texas). Dr. Chang discovered that PML was a substrate of the cell cycle regulatory kinase Cdk2 and these 9 serines represent the potential Cdk2 phosphorylation sites within PML.

We tested the PML 9 serine mutant for its ability to form PODs and IE72 affect on those PODs. SkBr cells were transfected with plasmids expressing either wild-type PML or the 9 serine mutant PML. **The PODs from cells transfected with the mutant PML were slightly smaller than normal however, upon cotransfection of pSG-IE72 and the pSG-9 serine mutant PML, we determined that IE72 could mediate POD disruption.** Therefore, none of these 9 serines are required for IE72 POD disruption function. We suggest IE72 regulation over PML and POD disruption is more complex and there may be multiple IE72 phosphorylation sites on PML and IE72 may facilitate the phosphorylation of PML by a cellular kinase.

IE72 mediates PML degradation through one of the 9 mutated serine residues in PML.

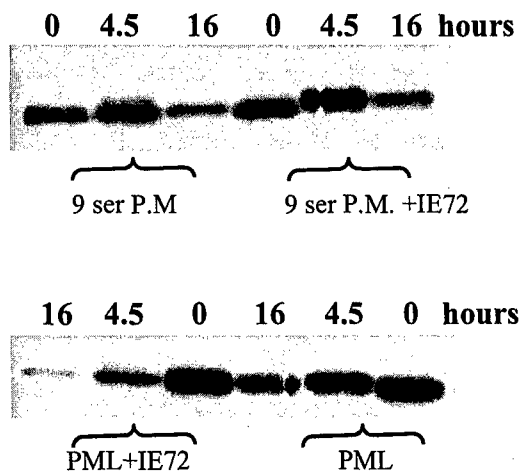
1. While analyzing the immuno-stained PML in cells co-expressing the PML-9 serine mutant and IE72, we observed that the **intensity of staining of mutant PML was greater** than wt PML, which suggests that more of the mutant PML is in the cell. It was also noted that there was **more cytoplasmic staining of the mutant PML.** One interpretation of these results suggests that upon IE72 mediated POD disruption, PML is translocated to the cytoplasm where it is degraded. This hypothesis would content that both wt and mutant PML are phosphorylated by IE72 contributing to POD disruption. However another IE72 mediated phosphorylation of PML is involved in the degradation of PML and the serine(s) involved in the later function is/are one of the 9 mutated.

2. **Further support of the above theory comes from the analysis of the protein profiles** between wt PML and the PML-9 serine mutant. These data show that IE72 mediates the loss of wt PML but not of the mutant PML. It is also interesting to note that the wt PML exhibits more bands upon coomassie-staining compared to the mutant PML which has two predominant bands. This banding pattern suggests that some of the 9 serines are phosphorylated *in vivo*. Finally the disappearance of one of the mutant proteins in the presence of IE72 may represent the phosphorylation of that protein by IE72. **Therefore it is possible that IE72 phosphorylates PML at multiple sites with differential results such as POD disruption and PML degradation. We may need to return to our tryptic peptide maps to identify and analyze other IE72 phosphorylated PML peptides in order to find one involved in POD disruption.**



3. Final support for the above hypotheses comes from pulse chase experiments in which wt and mutant PML have been over-expressed and metabolically labeled with S35 methionine followed by a chase with cold methionine. The following autoradiograms show the faster turnover of PML in the presence of IE72; whereas mutant PML turnover rate is not affected by IE72 (compare PML+IE72 0-4.5 hours to 9serine P.M. +IE72 0-4.5 hours).

IE72 can not mediate degradation of the 9 ser P.M



In conclusion, these data suggest that IE72 could mimic cdk2 thereby controlling the cell cycle and facilitating PML degradation during viral infection and mitosis.

The sumoylation of IE72 is not involved in POD targeting or in IE72 POD disruption function. (refer to manuscript #1 in the appendix for further details).

KEY RESEARCH ACCOMPLISHMENTS

1. The sumoylation of IE72 is not involved in POD targeting or in the IE72 process of POD disruption. **These data dispute the reported POD targeting function of SUMO-1 conjugation of PML.** (manuscript #1 accepted for publication pending minor modifications)
2. IE72 preferentially binds the SUMO-1-modified form of PML, which is the major POD-associated structural component. Therefore **we demonstrate that one function of sumoylated PML is to create new protein-interactive sites**
3. IE72 phosphorylates PML *in vivo and in vitro*
4. We have constructed an IE72 point mutant deficient for kinase activity
5. IE72 kinase activity is required for POD disruption

IE72 mediates the loss of SUMO-1-PML through **de-sumoylation**

6. IE72 kinase-activity is required for PML de-sumoylation. **Therefore IE72 induces a highly phosphorylated and desumoylated PML similar to the PML that predominates during mitosis.**
7. HCMV Δ IE72 can not mediate PML de-sumoylation
8. The affects of arsenic-treated PML (increase sumoylation and decrease phosphorylation) can reverse some of IE72 mediated PML deaggregation/POD disruption function
9. IE72 mediates the degradation of unconjugated-PML and this degradation involves one of 9 identified serines, which are potential cdk2 phosphorylation sites.

REPORTABLE OUTCOMES:

Manuscripts:

- 1.) The manuscript entitled "SUMO-1-modification of Human Cytomegalovirus (HCMV) IE1/IE72 is included in the appendix and has been accepted in the Journal of Virology for publication pending revisions.
- 2.) Another manuscript is in preparation to report on the interactions between IE72 and PML (figures 1-9 of this report).

Abstracts:

- 1.) I have received a travel honorarium and presented an award-winning poster at the Herpes International Workshop 2000 in Portland, Oregon. Entitled: The Characterization of SUMO-1-modified HCMV IE72
- 2.) I will present my data on the interactions between PML and IE72 at the next DOD meeting

Funding applied for based on work supported by this award:

- 1.) I have received a post-doctoral training grant from NIH to continue the study of PML mechanisms of growth suppression

Employment opportunity applied for based on training supported by this award:

- 1.) I have accepted a postdoctoral fellowship appointment within the laboratory of Dr. Michael Brattain at the Roswell Park Cancer Institute at Buffalo, New York.

CONCLUSIONS:

We conclude that the sumoylation of PML functions to create new protein interaction sites such as shown by the preferential binding of IE72 for SUMO-1 PML. POD-associated SUMO-1 modified PML is a substrate for IE72 and phosphorylation of PML contributes to the desumoylation of PML. The sum of our data, support our hypothesis and model: IE72 binds to and phosphorylates SUMO-1 modified PML, (POD associated PML) thereby contributing to PML de-sumoylation. This IE72 induced phosphorylation and de-sumoylation of PML is reversible as arsenic can partially reverse the IE72 affect. IE72 affects on PML posttranslation modifications are similar to the affects observed on mitotic PML by cellular kinase. The end result of the IE72 induced alterations on PML biochemistry is the loss of POD integrity/PML suppressor function.

We have narrowed the search for the phosphorylation site of PML, which is involved in PML sumoylation/POD disruption and in the process have discovered that IE72 also functions to mediate the degradation of PML via another phosphorylation event. We implicate one of 9 serines, to bwich is important in controlling the stability of PML. The definitive identification of these regulatory phosphoamino acid sites within PML will be useful is designing a PML-based therapeutic approach to the treatment of breast cancer. We conclude that IE72 mimics a mitotic cellular kinase therefore providing us with a valuable tool for the investigation into the mechanisms of PML tumor suppressor function.

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APPENDIX

1. Manuscript accepted for publication pending revisions
(38 pages)
2. Abstract presented at the Herpes International workshop (2000) Portland, Oregon
(1 page)
3. Summary Report from Mass Spectrometry Analysis of PML tryptic peptide
(1 page)
4. CV
(3 pages)

SUMO-1-Modification of Human Cytomegalovirus (HCMV) IEI/IE72

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Abstract

Human cytomegalovirus (HCMV) immediate-early protein, IE1/IE72 is involved in undermining many cellular processes including cell cycle regulation, apoptosis, nuclear architecture, and gene expression. The multifunctional nature of IE72 suggests that post-translational modifications may modulate its activities. IE72 is a phosphoprotein and has intrinsic kinase activity (Pajovic et al. 1997, Mol. Cell Biol. 17:6459-64). We now demonstrate that IE72 is covalently conjugated to Small Ubiquitin-like Modifier (SUMO-1). SUMO-1 is an 11.5 kDa protein that is conjugated to multiple proteins, and has been reported to exhibit multiple effects, including modulation of protein stability, subcellular localization and gene expression. Western blot with anti-IE72 of lysates from cells infected with HCMV or cells expressing IE72 reveal a covalently modified protein migrating at ~92 kDa, which is stabilized by a SUMO-1 hydrolase inhibitor. IE72 sumoylation was confirmed by analyses of immunoprecipitates of anti-IE72 and anti-SUMO-1 by Western blots with anti-SUMO-1 and anti-IE72, respectively. A point mutant of IE72 in which lysine 450 is changed to arginine is not SUMO-1-modified; lysine 450 is within a sumoylation consensus site (I,V,L)KXE. Both wild type IE72 and IE72_{K450R} target the nuclear PODs and result in their disruption. However, IE72_{K450R} is defective in complementation of IE72 deficient HCMV (CR208), suggesting that SUMO-1 modification of IE72 is important for viral replication.

INTRODUCTION

Human Cytomegalovirus (HCMV) is a member of the Herpesvirus family, exhibiting a narrow host range and a characteristic temporal cascade of gene expression in permissive cells. While HCMV poses a low threat to healthy individuals, it is life threatening to the immunocompromised, including prenatally infected newborns and AIDS patients (3, 30).

Primary transcripts from the major immediate-early region undergo alternative splicing to yield 5 gene products. There are two major spliced transcripts, IE1 and IE2 derived from the major immediate early region. The 1.95kb IE1 transcript is comprised of exons 1-4 and gives rise to the abundant IE72 gene product. This 491 amino acid protein is present throughout HCMV infection (42). The IE2 transcript is comprised of exons 1, 2, 3 and 5 and encodes IE86, which is a promiscuous trans-activator of both viral and cellular promoters. During infection, IE72 and IE86 are the first and most abundantly expressed proteins, and are required for the subsequent induction of the early and late genes. IE72 and IE86 contain a common transactivation domain encoded within exon 3, which encodes amino acids 25-85 of both proteins (37). IE86 and IE72 independently and synergistically activate heterologous promoters (8, 9, 16, 48). Cellular permissiveness for HCMV infection requires IE72 transactivation of the MIEP enhancer through the NF- κ B site (43). Thus, both IE86 and IE72 are major gene regulatory factors which play essential roles in HCMV infection.

We have discovered that IE72 is a viral kinase capable of phosphorylating itself, as well as E2F1-3 and the pocket proteins p107 and p130, but not E2F4 or 5 or pRb (36). The important role that IE72 plays in HCMV lytic growth is underscored by the fact that a recombinant virus bearing a deletion of exon 4 in the MIE region is severely impaired for replication at low MOI (30). This block in DNA replication correlates with a defect in the accumulation of ppUL44, an early gene product required for viral DNA polymerase, and failure to form DNA replication compartments, which may be related to a failure to disrupt the nuclear structures referred to as PML oncogenic domains (PODs) (2), nuclear domain 10 (ND10) or nuclear dots (ND). These defects can be corrected when IE72 is supplied in *trans* (15).

IE72 is involved in viral effects on numerous cellular processes including gene regulation, cell-cycle progression, signal transduction, POD dispersal, and apoptosis (2, 25, 27, 50, 52). Post-translational modifications are common mechanisms for the regulation of multi-functional proteins. Our studies have determined that IE72 is auto-phosphorylated (36) and is also phosphorylated at distinct sites by a cellular kinase(s) (Himmelheber, Azizkhan-Clifford, Manuscript in Preparation). The present investigation demonstrates that IE72 exhibits a novel post-translational modification in which the small ubiquitin-like modifier (SUMO-1) is covalently attached to lysine 450 of IE72.

SUMO-1 (also known as sentrin, GMP1, PIC1, and Ub11, or in yeast as SMt3), an ubiquitin-like protein sharing 48% homology with ubiquitin (5), functions as an important reversible protein modifier. Since its discovery in 1996, a growing list of proteins have been reported to be SUMO-1-modified (51, for review); many SUMO-modified proteins are associated with PODs. The major POD structural component,

PML, was first discovered in acute promyelocytic leukemia patients exhibiting a chromosomal translocation (15:17) that fuses the retinoic acid receptor to PML (7, 10, 14, 22). PODs have more recently been identified as sites of cellular and viral replication processes (26). While POD organization has been linked to cell transformation, PODS are the target for a variety of viral proteins, and the loss of POD organization is thought to be important in the viral infection cycle (1, 18, for review). SUMO-1 conjugation accounts for specific effects, including altered stability, gene regulation, sub-cellular localization and protein-protein interactions (11, 19, 24, 39). For example, the SUMO-1 conjugation of promyelocytic leukemia protein (PML) has been reported to function as a POD targeting signal (34), whereas SUMO-1 modification of RanGAP1 enables its interaction at the nuclear pore complex and SUMO-1 modification of p53 increases its transactivation activity. In this study, we have mapped the site of SUMO modification on IE72 to lysine 450 and have demonstrated that IE72 sumoylation is not involved in the ability of IE72 to target or disrupt the nuclear structures known as PODs, nor does it appear to significantly alter IE72 stability or trans-activation activity. Although the precise mechanism remains to be determined, IE72 sumoylation is important in HCMV replication based decreased ability of IE72_{K450R} to complement IE72-deficient HCMV.

MATERIAL AND METHODS

Cell culture. Glioblastoma cells (U373-MG) and human foreskin fibroblasts, HFF, were used for HCMV infection and for the expression of HCMV IE72. Cells were cultured in Dulbecco Modified Eagle's Media (DMEM), supplemented with 2 mM L-glutamine, 10% fetal calf serum and penicillin/streptomycin (100 µg/ml), in a humidified 10% CO₂ incubator at 37°C. The human embryonic kidney cell line, 293, was used to propagate recombinant Adenovirus (49). HCMV was propagated in HFF as described (35).

Calyculin A (Sigma) was prepared as a 10 µM stock solution in DMSO; the final concentration for cell treatment was 0.1 µM.

HCMV infection. HCMV stocks were prepared from HFF cells infected at a multiplicity of infection (M.O.I.) of 0.1 plaque forming units per cell. An inoculum in 2% heat-inactivated FBS/DMEM was allowed to adsorb to cells for 4-6 days. Media was collected from cells displaying approximately 75% cytopathic effects and centrifuged at 3,000 rpm for 10 minutes to remove cell debris. Aliquots of the stock supernatant were frozen at -90°C. Plaque assays were performed and titers calculated as plaque forming units per ml. IE72-deficient HCMV from which the exon 4 region was deleted (CR208) was generously supplied Richard Greaves (15).

Adenovirus infection. The recombinant adenoviruses, AdLacZ (rAd35) and AdIE72 (rAd31) were kindly provided by Gavin Wilkinson (49). The E. coli lacZ and the HCMV IE72 cDNA are expressed under the control of the HCMV major immediate-early promoter. HFF cells were infected with adenovirus at an M.O.I. of 30 pfu/cell, in a minimal volume of 2% heat-inactivated FBS/DMEM for at least 24 hours.

Retrovirus infection. The amphotrophic Phoenix cells were used to package recombinant retroviruses according to instructions found at

[HTTP://WWW.STANFORD.EDU/GROUP/NOLAN/](http://www.stanford.edu/group/nolan/).

5×10^6 Phoenix cells/10 cm culture dish were plated 24 hours prior to infection. Chloroquine (25 μ M) was added 5 minutes before Phoenix cells were transfected with 20 μ g of the retroviral plasmid construct by the calcium phosphate co-precipitation method (4). Cells were incubated at 32°C overnight, followed by a wash in phosphate buffered saline (PBS) and an additional 24 hour incubation at 32°C in media containing 10% FBS. The media was cleared and used immediately to infect HFF that were plated the night before at 1.5×10^5 cells/well in 6-well dishes and incubated 6-8 hours in sodium phosphate-free DMEM supplemented with 10% dialyzed FBS. Retroviral supernatant (1ml/well), supplemented with 5 μ g/ml polybrene, was added to the cells and incubated at 32°C for 24 hours. Cells were then washed and incubated in DMEM/10% FBS at 37°C for 24 hours.

Plasmids. The pSG5 plasmids containing cDNA encoding wild-type IE72 or deletion mutant Δ aa 421-486 were kindly provided by John Sinclair (17). The IE72 point

mutant in which lysine 450 was substituted for arginine was constructed using pSG-IE72 as template and the two step PCR mutagenesis technique. The first round consisted of two separate PCR reactions to generate the 5' and 3' ends of the gene with the desired mutation. The 5' end was made using the primers 5'-AGCAATTCGGATCCATGGAGTCCTCTGCCAAGAG-3' (5' IE-1 primer) and 5'-GTGTCTGTCAGGTCTGAGCCA-3'. The 3' end was made with the primers 5'-ACGAATTCGGATCCTGATTAGTGGGATCCATAACAGTAAC-3' (3' IE-1 primer) and 5'-TGGCTCAGACCTGACAGACAC-3'; pSG-IE72 was the template in both reactions. The products from these PCR reactions were gel purified and used as templates for the second round PCR reaction with both the 5' and 3' IE-1 primers. The final 1.5 kB fragment was digested with Afl II and PflM I and the resulting 530 bp fragment was ligated into a pSG-IE72 backbone which had been digested with the same enzymes. The final recombinant plasmid was verified by sequencing. The IE72 wild type and mutant constructs, as well as the DHFR reporter plasmids employed for luciferase assays, have been previously described (6, 25).

The LXSG retroviral vector was obtained from Dusty Miller. LXSG was constructed from LXS_N (29) by replacement of the neomycin phosphotransferase cDNA (neo) with EGFP (a variant of green fluorescence protein, Clontech). LXSG-IE72 was created by subcloning the blunt-ended SacI/BamHI IE72 cDNA fragment from pRSV-IE72, into the blunt-ended XhoI and BamHI sites of LXSG vector, downstream of the Moloney murine leukemia virus promoter (MMLV LTR). LXSG IE72_{K450R} was constructed using Stratagene's Quik Change site-directed mutagenesis kit. Briefly, the oligonucleotide primers used to construct pSG-IE72_{K450R} were annealed to the LXSG

IE72 template. Using the nonstrand-displacing action of Pfu Turbo DNA polymerase, the mutagenic primers were extended, resulting in nicked circular strands. The non-mutated methylated parental DNA template was digested with DpnI and the circular, dsDNA was transformed into competent cells.

Antibodies. Polyclonal antiserum directed against IE72 and IE86 was generated by immunizing rabbits with a polypeptide corresponding to amino acids 12-25 (pAb543). Polyclonal antibody that specifically recognizes IE72 (1-2) was obtained from Dr. Jay Nelson. The monoclonal antibody, BS500, which recognizes an epitope in IE72 (amino acid residues 389-425), was generously provided by Dr. Bodo Plachter (Johannes Gutenberg-Universitat Mainz). Monoclonal antibody directed against SUMO-1 was purchased from Zymed Laboratories. Dr Roel Van Driel (University of Amsterdam) generously supplied us with a monoclonal antibody directed against PML (5E10) (45).

Western Blotting and immunoprecipitation analysis. Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) or in 50 mM Tris, pH 6.8/2% SDS/10% glycerol supplemented with 20 μ M N-ethylmaleimide (NEM) followed by centrifugation at 4°C at 14,000 g for 20 minutes. The supernatant was incubated with the appropriate primary antibody and protein A or G- Sepharose for 3-4 hours at 4°C. The beads were collected and washed in RIPA buffer three times, before boiling in SDS buffer for analysis by Western blotting. Proteins were separated by SDS-PAGE (8%) and electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 3% fat-free dried milk in PBS

supplemented with 0.1% Tween 20, then incubated with the appropriate primary antibody for one hour at room temperature. Subsequently, membranes were incubated with a secondary antibody conjugated with horseradish peroxidase. Each of the incubation steps was followed by 3 washes for 5 minutes in PBS/0.1% Tween. Development was performed with Super Signal according to the manufacturer's protocol (Pierce).

Indirect immunofluorescence analysis. U373 cells were grown over night on cover slips in 35mm 6 well plates. 48 hours post transfection, cells were washed in PBS and fixed with 2% formaldehyde for 15 minutes at room temperature. Cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min, followed by incubation for 1 hour at room temperature with the appropriate primary antibody (1/500 dilution of the polyclonal antibody directed against IE72; 1/100 dilution of the monoclonal antibody directed against PML). After 3 washes in PBS-Triton, cells were incubated at room temperature, with the appropriate secondary antibodies: anti-mouse tetramethyl rhodamine isothiocyanate (TRITC) and anti-rabbit FITC-conjugated secondary antibodies (Jackson Labs). Cells were mounted using Vectashield mounting medium (Vector Laboratories) and analyzed with a fluorescence microscope (Carl Zeiss, Inc) equipped with the appropriate optics and filter modules and results were recorded with a digital camera (SPOT, Diagnostic Instruments Inc).

Complementation assay. Phoenix cells were transfected with 20 µg each of LXSG empty vector, LXSG-IE72 or LXSG-IE72_{K450R} by the calcium phosphate co-precipitation method and cultured at 32°C overnight. 24 hours post-transfection, cells

were washed and cultured overnight in 5 ml DMEM for 24 hours at 32°C. 48 hours post-transfection, retrovirus was collected and used immediately to infect HFF plated at 1.5×10^5 /well on a 6-well dish. Forty-eight hours later, cells were infected with 200 μ l of a $1:10^3$ dilution of the HCMV Δ IE72 (CR208) stock @ 1.6×10^5 pfu/ml, followed by addition of an agarose overlay. Cells were monitored daily for plaques, whereupon they were fixed, stained and counted.

RESULTS

Western Blot analysis detects two major forms of IE72. Western blot using anti-IE72 (monoclonal antibody against amino acid residues 389-425), which does not cross react with IE86, revealed two major protein species in HCMV infected HFF cells. A major protein that migrates with an apparent molecular mass of 72 kDa, as well as an additional protein that migrates at approximately 92 kDa were detected (Figure 1). This apparent 20 kDa molecular weight increase in IE72 is consistent with SUMO-1 conjugation, which induces a characteristic 20 kDa mobility shift (11, 23). The 92 kDa form of IE72 is highly sensitive to conditions of lysis, and is only seen if cells were lysed under denaturing conditions, such as 2% SDS or 6M guanidine HCl. Under these conditions, as much as 1/3 of the total IE72 is in the 92 kDa form. Western blot analysis was performed on lysates from several different cell types, including HFF and U373 cells infected with either HCMV or recombinant adenovirus expressing IE72 or transduced with a retrovirus expressing IE72. Several different monoclonal and polyclonal antibodies were found to detect the 92 kDa form of IE72. There was no detection of either form of IE72 in U373 or HFF cells that were infected with CR208, a mutated version of HCMV that does not express IE72, or in cells infected with the recombinant adenovirus expressing β -galactosidase or transduced with the control retrovirus empty vector (LXSG).

Detection of the ~92 kDa IE72 protein after immunoprecipitations. The 92 kDa form of IE72 was not detected in immunoprecipitations performed in RIPA buffer. Since the SUMO-1 conjugation of proteins is readily hydrolyzed under non-denaturing conditions, N-ethylmaleimide (NEM), a potent inhibitor of deubiquitinating enzymes, which also inhibits SUMO-1 hydrolase activity (46), was added to immunoprecipitations. Cell lysates from U373 cells transfected with an IE72 expression vector were immunoprecipitated in the presence or absence of 20 μ M NEM with a polyclonal antibody to IE72 (pAb543). Western blot of these IE72 immunoprecipitates with a specific anti-SUMO-1 antibody detected a protein that co-migrated with the ~92 kDa IE72 protein (Figure 2). The 92 kDa form was not detected in immunoprecipitates in the absence of NEM. Thus, a specific SUMO-1 hydrolase inhibitor protected the highly labile form of IE72, suggesting that the higher molecular weight species may be sumoylated.

Detection of the ~92 kDa form of IE72 by an antibody directed against SUMO-1. U373 cells were infected with a recombinant adenovirus (RAd) expressing either β -galactosidase or IE72. Cells were harvested in 50 mM Tris, pH 6.8/2% SDS/10% glycerol, boiled 10 minutes and diluted 1:8 in PBS containing NEM, followed by addition of anti-SUMO-1 antibody (Fig. 3A) or anti IE72 (pAB543) (Fig. 3B); immunoprecipitates were subjected to Western blot analysis using the reciprocal antibodies, anti- IE72 (BS500) (Fig. 3A) or anti SUMO-1 (Fig. 3B). The ~92 kDa protein immunoprecipitated by anti-SUMO-1 cross-reacted with anti IE72 (Fig. 3A), and that immunoprecipitated by anti-IE72 cross reacted with anti-SUMO-1 (Fig. 3B). The

reciprocal immunoprecipitations of the 92 kDa species with anti-SUMO-1 and anti-IE72 provide strong evidence for the 92 kDa species of IE72 resulting from its SUMO-1 modification.

Relationship between SUMO-1 conjugation and phosphorylation of IE72.

Phosphorylation of PML, c-jun, and p53 appears to negatively regulate their SUMO-1 modification (13, 31, 34). Calyculin A (a potent inhibitor of serine/threonine phosphatases 1 and 2A), was used in the studies above to investigate the involvement of phosphorylation in sumoylation; therefore, we treated U373 cells stably expressing IE72 with either 0.1 μ M Calyculin A dissolved in DMSO or with an equal volume of DMSO. Cells were lysed by boiling in SDS sample buffer, and the extracts were analyzed by Western blot with anti IE72 for the presence of SUMO-modified IE72. Treatment of cells with calyculin A for as little as 30 minutes significantly decreased the amount of sumoylated IE72, and treatment for 1 hour led to complete loss of sumoylated IE72, without affecting the levels of unconjugated IE72 (Figure 4). These data suggest that serine/threonine phosphorylation of IE72 and/or proteins acting upon IE72, suppresses the formation of SUMO-1-IE72 conjugates. We are currently identifying the phosphoamino acid(s) involved in the regulation of IE72 sumoylation.

Identification of the site of SUMO-1 modification in IE72. Having determined that IE72 is SUMO-1-modified, we sought to identify the region of IE72 involved in this modification. Lysates of cells transiently transfected with either wild-type IE72 or various C-terminal truncation mutants of IE72 were analyzed by anti-IE72

Western blot. While a SUMO-modified form was clearly evident with wild-type IE72, no evidence of SUMO-1 modification could be detected on a mutant with the C-terminus deleted (IE72 Δ 420-486) (data not shown). Analysis of the amino acids within this C-terminal region revealed a consensus sequence for sumoylation $_{449}(I/L/V) \underline{K}XE_{452}$ (Figure 5A). We constructed a point mutant of IE72 in which lysine 450 was substituted with arginine (IE72_{K450R}). U373 cells were transfected with pSG-IE72 or pSG-IE72_{K450R}. Cell lysates were subjected to anti-IE72 Western blot (Figure 5 B) and the 92 kDa band seen with wild-type IE72 is not seen with the point mutant. In a similar experiment, cells were labeled with ^{35}S methionine, followed by immunoprecipitation with anti-IE72, SDS/PAGE and autoradiography. The 92 kDa radiolabeled band is only seen in the cells transfected with wild type IE72 and not with the K450R mutant (Figure 5C). Thus, IE72_{K450R} is deficient in SUMO-1 modification.

IE72 sumoylation is not involved in POD targeting nor POD disruption.

Having determined that IE72 is SUMO-1-modified and having identified the lysine residue involved in this conjugation process, we proceeded to address the functional implications of this novel post-translational modification.

SUMO-1 modification of PML has been reported to be involved in its targeting to PODs (12, 23, 34, 44). Since HCMV IE72 is localized in and disrupts PODs (2, 50), we tested the hypothesis that SUMO-1 conjugation of IE72 was responsible for POD localization. U373 cells grown on cover slips were transfected with either wild-type IE72 or IE72_{K450R} and double immunostained for PML and IE72. We observed no differences in the POD localization or POD disruption in the sumoylation-deficient IE72 when

compared to the wild-type IE72 (Figure 6). Therefore, we conclude that SUMO-1 is not responsible for the ability of IE72 to target and subsequently disrupt PODs.

Complementation of CR208 with IE72_{K450R}. IE72-deficient HCMV (CR208), was used to test the significance of IE72 conjugation to SUMO-1 as it relates to HCMV replication. HFF were transduced with a retrovirus empty vector (LXSG) or a retrovirus that expresses wild type IE72 or IE72_{K450R}. The inset shows the equivalent levels of expression IE72_{K450R} (left lane) or IE72 (right lane) by Western blot. These cells were then used to test the ability of the expressed protein to complement the IE72 deficient virus. The relative numbers of plaques are shown in Figure 7; IE72_{K450R} results in significantly lower levels of complementation as compared to wild-type IE72 (Student t test, $P < 0.01$). The sumoylation deficient IE72 consistently exhibited between 40-50% fewer plaques compared to the wild-type IE72. Our results demonstrate that IE72 that cannot be SUMO-1 modified is impaired in its ability to complement infection by IE72-deficient virus, suggesting that SUMO-1 modification of IE72 plays a role in HCMV replication.

DISCUSSION

IE72 has two major forms of differing molecular weights on SDS/PAGE. The slower migrating form migrates at an apparent molecular weight of approximately 92 kDa. The increase in apparent molecular weight is approximately 20 kDa, a size consistent with a single SUMO-1 conjugation. The amount of the 92 kDa protein is increased in the presence NEM, a SUMO-1 hydrolase inhibitor. This 92 kDa form of IE72 was immunoprecipitated with antibodies directed against either IE72 or SUMO-1. There is a sumoylation consensus sequence in the C-terminus of IE72, (I/V/L)**K**XE, where **K** represents lysine 450. A mutated protein, in which lysine 450 was substituted with arginine, was incapable of being SUMO-1 modified. Using this sumoylation deficient IE72, we proceeded to verify that the SUMO-deficient IE72 was able to complement infection by IE72-deficient HCMV (CR208) only 50% as well as wild type IE72, suggesting that sumoylation is involved in the function of IE72 in viral replication.

Further corroborative evidence for the sumoylation of IE72 comes from the observation that IE72 can be SUMO-conjugated in cells that overexpress SUMO-1 (19, 32).

One of our initial hypotheses to explain the role of SUMO conjugation of IE72 was that SUMO-1 served as a POD-targeting signal for IE72. This idea was based on the association of IE72 with PODs (2, 50) and the reports that SUMO-1 conjugation to promyelocytic leukemia protein, PML (the major nuclear POD structural protein), served as a POD targeting signal (34). However, we found that the SUMO-1 deficient IE72 and

wild type IE72 had an equal ability to target and disrupt PODs (Figure 6). More recent data demonstrate that SUMO-1-conjugation to PML may not be required for PML translocation to the PODs; however, it is necessary for the recruitment of other POD associated proteins and for the maintenance of POD integrity (21).

Our studies have shown that IE72 is phosphorylated at distinct sites in its C-terminal region through autophosphorylation and also through phosphorylation by a cellular kinase (36, Himmelheber and Azizkhan-Clifford, manuscript in preparation). Our data showing that inhibition of ser/thr protein phosphatases by calyculin A results in a decrease in the SUMO-1-modified form of IE72 suggest that phosphorylation may inhibit SUMO-1 conjugation. The sumoylation site is adjacent to the region of autophosphorylation and we are presently analyzing the role of phosphorylation at specific sites in IE72 sumoylation. It is highly probable that the post-translational modifications of phosphorylation and sumoylation play an important role in IE72 interaction with other gene regulatory proteins because IE72 has been shown not to bind DNA directly, implicating protein-protein interactions in IE72 gene regulatory function.

The failure of the sumoylation-deficient IE72 to complement IE72-deficient HCMV may be the result of altered stability of IE72_{K450R}. Although we have no direct evidence to suggest this mechanism, we have found that phosphorylation-deficient IE72 is significantly less stable than wild type (data not shown), and the inverse relationship between phosphorylation and sumoylation could suggest an indirect role of sumoylation in stability. SUMO-1 modification of a protein can serve to stabilize it against proteosomal lysis through competition for a lysine residue that is used for both sumoylation and ubiquitination (e.g. I κ B α) (11). Additional experiments are required for

a comprehensive understanding of the role of phosphorylation and sumoylation in IE72 stability.

A review of the growing list of SUMO-1-modified or SUMO-1/Ubc9 interactive proteins suggests that SUMO-1 is important in numerous cellular processes (33, for review, 51) including:

apoptosis (p53, Mdm2, Daxx, I κ B alpha, FAS/apolipoprotein-1, TNFR1), viral oncogenesis (E1A, papilloma E1, HCMV IE1 and IE2), transcription and cell cycle progression (ETS-1, TEL, CBP/p300, c-jun, glucocorticoid and androgen receptors, E2A, ATF2, p53, p73, HIPK2, MITF, and Wilm's tumor gene product), POD formation (PML, Sp100), centromere function (Cbf3, MITF), nuclear import (RanGap1), and maintenance of genome integrity (topoisomerases, p53, Wrm Rad 51, 52). From this partial list of sumo-interactive proteins, we can predict that SUMO-1 plays a role in transcription. The sumoylation motif is identical to a common motif within the negative regulatory regions of multiple factors (20). In accord with these data, sumoylation negatively regulates the transcription of c-jun and steroid receptors (31, 38), while others report that sumoylation increases transactivation function of p53 and HCMV IE86 (39). Using DHFR-luciferase, a promoter that is significantly transactivated by IE72 alone (25, 47), we found that sumoylation of IE72 reduces the trans-activation of DHFR-luciferase by at most two-fold compared to the wild type. The function of SUMO-modification of IE72 in transactivation needs to be further explored.

The SUMO-1 yeast homologue was identified as a suppressor of mutations in Mif2 (mitotic instability factor 2), a protein thought to be the equivalent of the mammalian centromere protein, CENP-C (28). Therefore SUMO-1 may play a role in

meiosis and/or mitosis control. We and others have found specific effects of IE72 on the cell cycle (27, 40). Since free SUMO-1 is limiting in the cell, SUMO-1 deconjugation is required to release SUMO-1 for additional protein modifications (11). SUMO-1 conjugation and de-conjugation must be in balance for controlled cell growth (41). Thus, considering the abundance of IE72 in infected cells and the extent of its SUMO- modification, sumoylation of IE72 could exert a global modulation of SUMO-1, which could regulate diverse genetic programs.

The SUMO-1 modification of IE72 may play a role in the precise temporal cascade of gene expression exhibited during HCMV infection. Considering the rapidly expanding list of proteins modified by SUMO-1, the contribution of sumoylated IE72 to HCMV replication may be complex. For example, SUMO modification may affect the ability of IE72 to interact with other factors to modulate effects on the cells that are essential for viral replication. Further experimentation is required to determine the possible effects of sumoylation of IE72 in its ability to modulate gene expression, cell cycle control and apoptosis.

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Figure Legends

Figure 1. Two forms of IE72 of apparent molecular weights of 72 and ~92 kDa can be detected in HCMV-infected cells. HFF cells were infected with HCMV at the indicated M.O.I. Cell lysates were prepared by direct lysis in SDS sample buffer and subjected to Western Blot with anti-IE72 (BS500). In addition to the major IE72 band migrating at 72 kDa, a significant band cross-reacting with IE72-specific antibody was detected at ~92 kDa. Mock-treated cells do not show either protein.

Figure 2. Addition of N-ethylmaleimide (NEM) to immunoprecipitation reactions allows detection of the ~92 kDa form of IE72. Cell lysates from U373 cells transfected with pPSG-IE72 were immunoprecipitated with an anti-IE72 polyclonal antibody in the presence or absence of NEM, a specific SUMO-1 hydrolase inhibitor. Western Blot with anti-IE72 (monoclonal) revealed the presence of the ~92 kDa protein only in the lysates treated with NEM.

Figure 3. Reciprocal co-immunoprecipitation of SUMO-1 and IE72. **A.** U373 cells were infected with a recombinant adenovirus expressing either β -gal (lanes 2,4) or IE72 (lanes 1,3). Untreated lysates (lanes 3,4) or lysates immunoprecipitated with anti-SUMO-1 antibody in the presence of NEM (lanes 1,2) were resolved by SDS/PAGE (lanes 3,4) and subjected to Western Blot analysis using an antibody against IE72. **B.** U373 were infected with Ad IE72 (lane 1) or Ad β -gal (lane 2). 48 hours after infection, cells were harvested in 2% SDS buffer, boiled 10 minutes and diluted in PBS containing

NEM to allow anti-IE72 immunoprecipitation of IE72. Western blot analysis of the precipitates was performed with anti-SUMO-1 antibody. Lanes 3 & 4. HFF were either HCMV infected (lane 3) or mock-treated (lane 4). Cells were lysed in RIPA buffer supplemented with NEM. Immunoprecipitations were performed with an antibody against IE72 and an anti-SUMO-1 antibody was used for the Western Blot.

Figure 4. IE72 sumoylation is negatively regulated by its phosphorylation. U373 cells stably expressing IE72 were treated with either DMSO or 20 μ M Calyculin A (a phosphatase inhibitor) dissolved in DMSO. At the indicated time points, cells were harvested by direct lysis and boiling in SDS sample buffer. An antibody directed against IE72 was used for Western blot.

Figure 5. IE72 lysine 450 is SUMO-1 conjugated. **A.** The C-terminus of IE72 contains a sumoylation consensus motif (V/I/L)KXE, implicating lysine 450 as the SUMO-1-conjugated residue. **B&C.** A point mutation was made substituting lysine 450 with arginine (IE72_{K450R}). **B.** U373 cells were transfected with pPSG-IE72 or pPSG-IE72_{K450R}. Cell lysates were subjected to immunoprecipitation with anti-IE72, followed by Western Blot with anti-IE72 in the presence of NEM and detection using a chemiluminescence reagent. **C.** Cells were metabolically labeled by addition of ³⁵S methionine to the media for 2 hours, followed by immunoprecipitation of IE72 in the presence of NEM, and SDS-PAGE and autoradiography.

Figure 6. The SUMO-1 modification of IE72 is not involved in POD targeting or disruption. HFF were transfected with either pPSG-IE72 or pPSG-IE72_{K450R}. 48 hours post transfection, cells were fixed, and co-immunostained with anti-IE72 and anti-PML antibodies. Secondary antibodies were tagged with Rhodamine (Panels A,B) and FITC (Panels C,D), respectively. In cells that do not express IE72, PML is localized within sub-nuclear structures referred to as Promyelocytic Oncogenic Domains (PODs) (Panels C,D). In cells not expressing IE72 (Panel A,B, unstained cells), PODs are clearly seen as nuclear dots of about 20-30 per cell (Panel C,D), whereas cells transfected with IE72 exhibit POD disruption (Panel C, arrows). Cells expressing IE72_{K450R} (Panel B, stained cells) exhibit POD disruption (Panel D, arrows) equivalent to that seen with wild-type IE72. Careful examination of the immuno-staining reveals PML localization in nucleoli in untransfected and transfected cells whereas IE72 is excluded from these structures.

Figure 7. IE72 that cannot be SUMO-1-modified is defective for complementation by IE72 mutant HCMV. HFF were transduced with a retrovirus vector (LXSG) or a retrovirus that expresses wild-type IE72 or IE72_{K450R}. 48 hours later, cells were infected with HCMV lacking IE72 (CR208) using a 1:10³ dilution of a 1.6x10⁵ pfu/ml viral stock and overlaid with 0.6% low melting agarose. Plaques were counted 9 days post HCMV infection. The inset shows the results of a Western blot of cells transduced with either IE72_{K450R} (left lane) or IE72 (IE72).

92 kDa →

72 kDa →



CMV MOI :

40

20

10

5

0

+ NEM - NEM



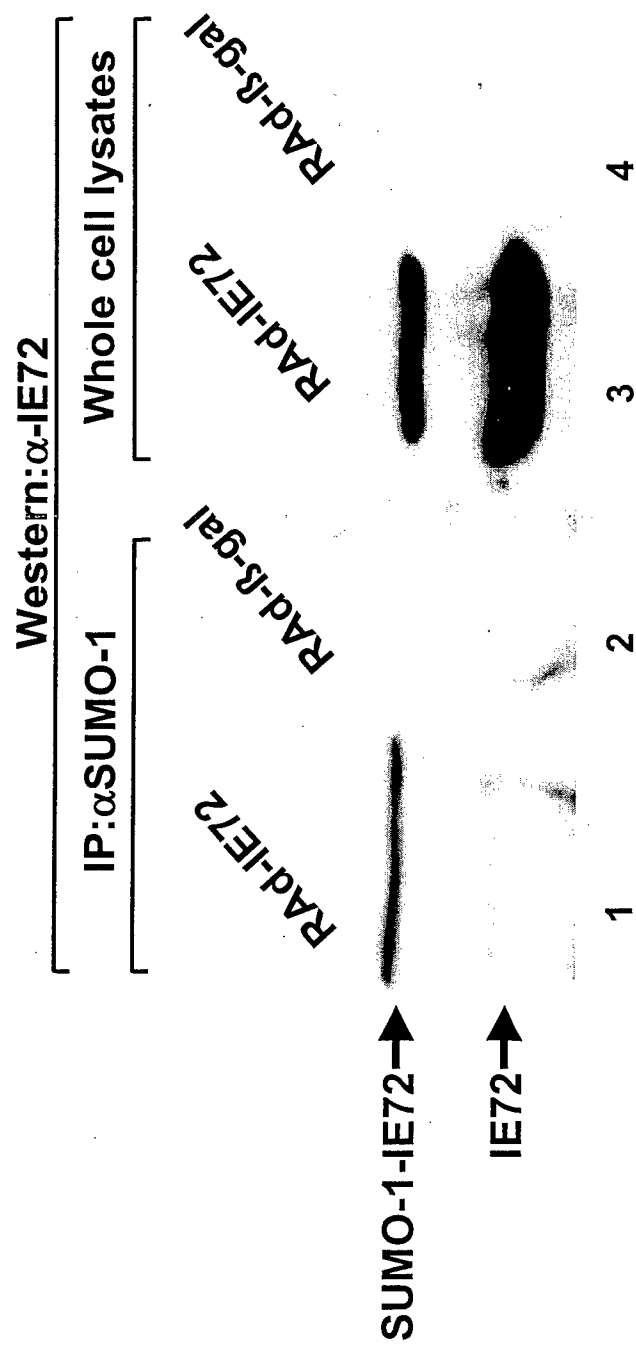
~92 kDa



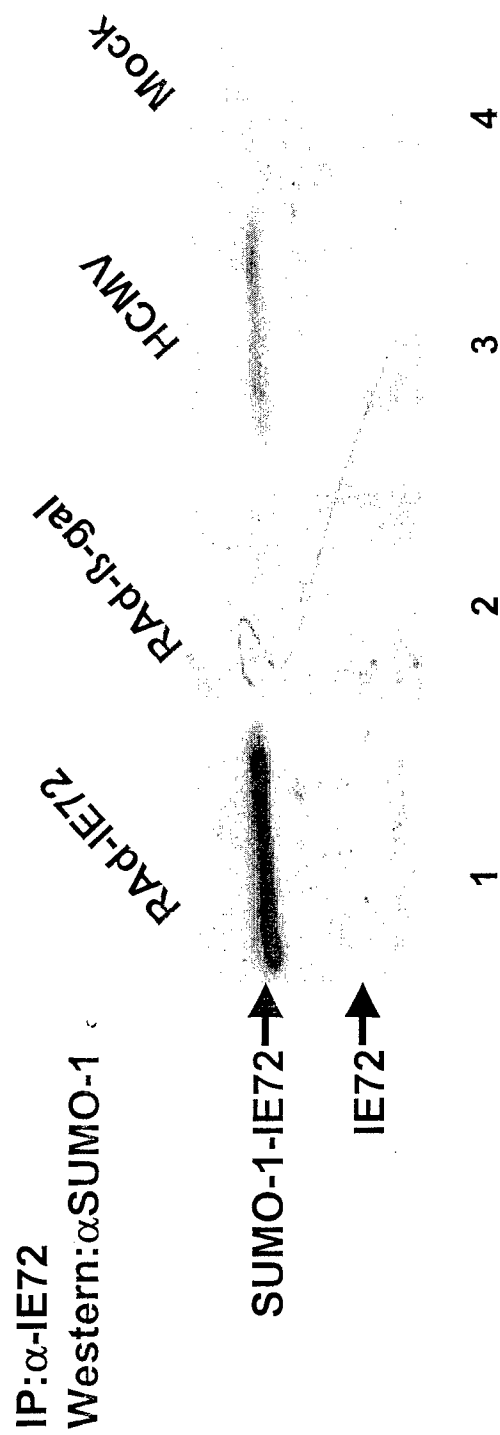
IE72



A



B





A

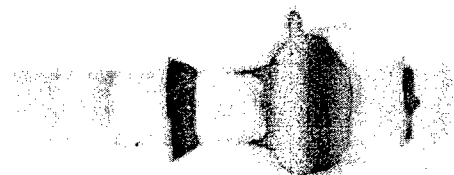
IE72 amino acids 421-486:

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EIEEVAPEEEEDGAEEPTASGGKSTHPMVTRSKADQ

B

wtIE72

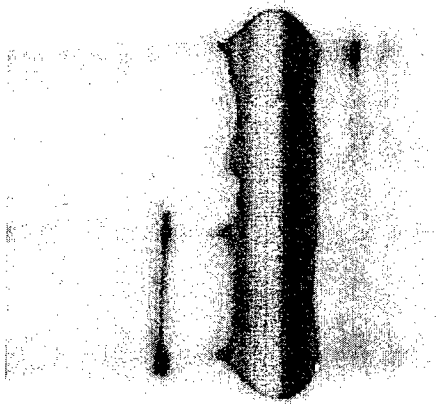
IE72^{K450R}



C

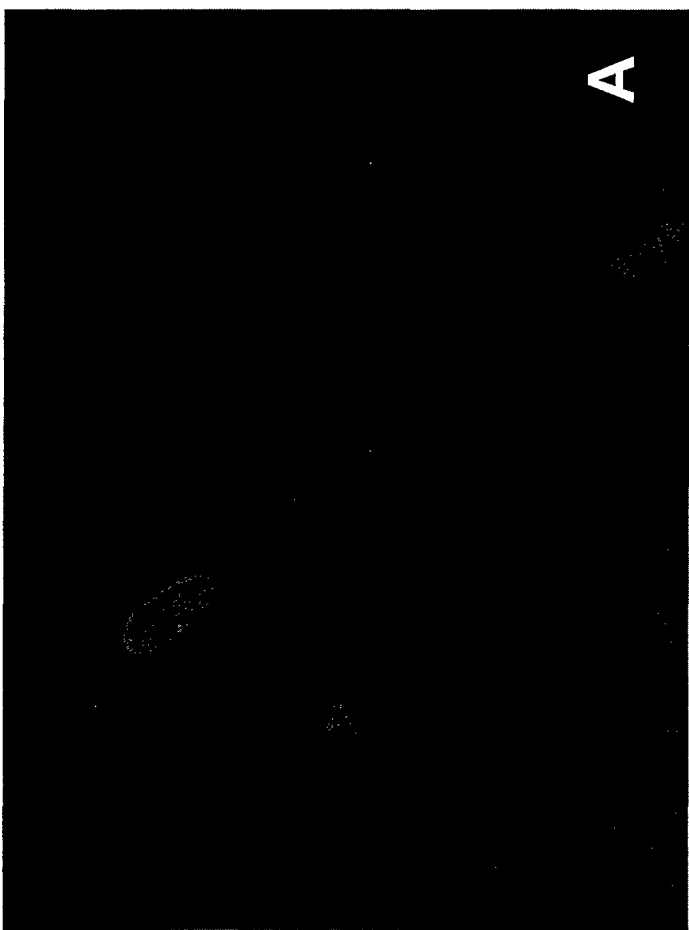
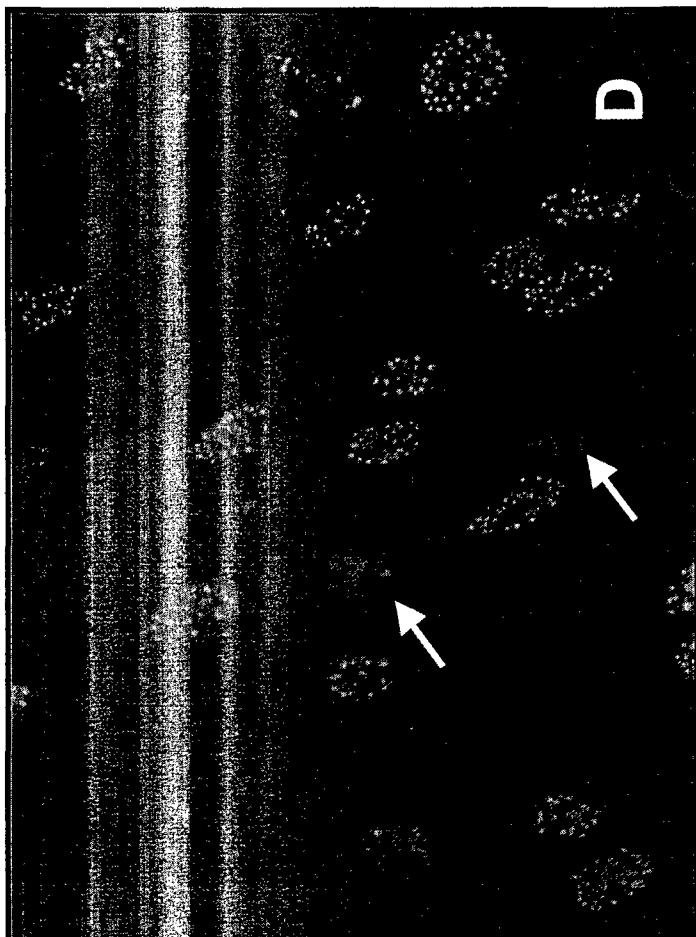
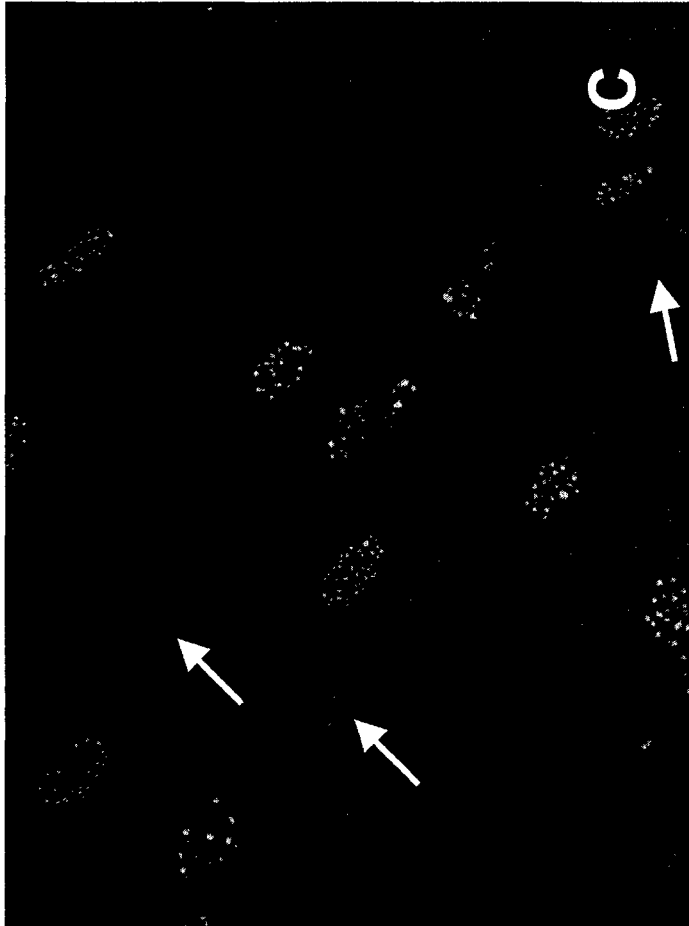
wtIE72

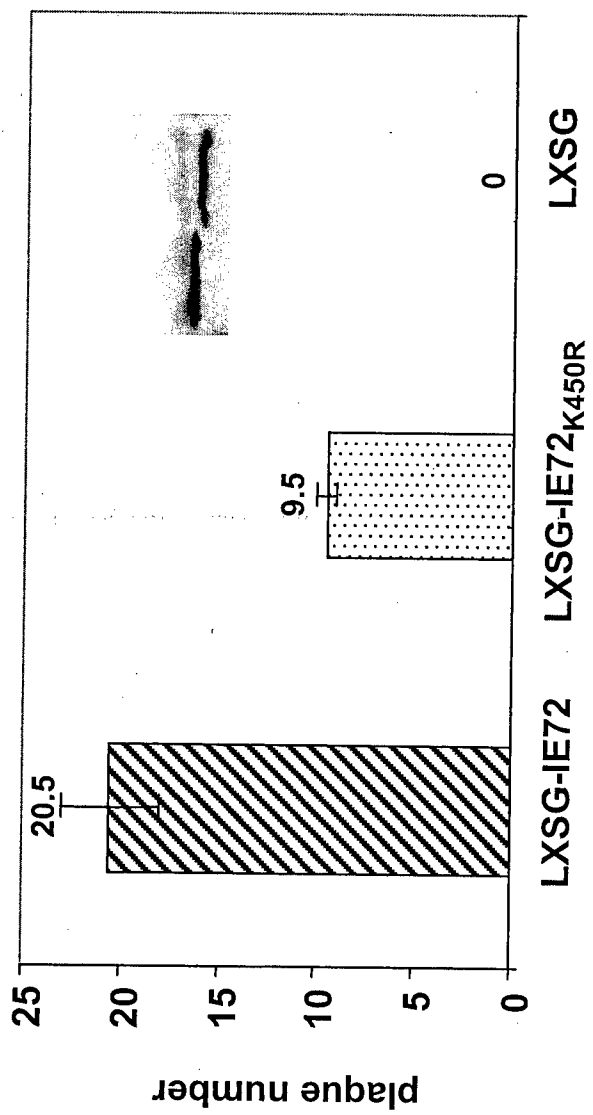
IE72^{K450R}



▲ SUMO-IE72 ▲

▲ IE72 ▲





Abstract

HCMV IE72 is a unique viral kinase, which is involved in many cellular processes including cell cycle regulation, apoptosis, nuclear architecture rearrangements, regulation of key G1 proteins, and gene expression, suggesting that IE72 may be highly modified to allow for the regulation of its multifunctional nature. The phosphorylation of IE72 is well established. We now demonstrate that IE72 is covalently SUMO-1-conjugated. Small Ubiquitin-like Modifier (SUMO) is an 11.5 kDa protein, which has been reported to modulate protein stability, subcellular localization and regulation of gene expression. Anti-IE72 Western blot analyses of SDS-PAGE resolved lysates from cells infected with HCMV or cells expressing IE72 reveal a covalently modified IE72 protein migrating at 92 kDa. This protein can be immunoprecipitated and specifically recognized by an anti-SUMO-1 antibody. Lysine 450 of IE72 corresponds to the SUMOylation consensus site (Ψ KxE), and a point mutant (Lysine 450 to Arginine) is not SUMO-1 modified. We are currently using this lysine 450 mutant (IE72 Δ SUMO) to determine the role of SUMO-modification in IE72 function. Both wt IE72 and IE72 Δ SUMO target the nuclear POD structures and result in their dispersal. Both wt IE72 and IE72 Δ SUMO upregulate gene expression from the DHFR promoter; however the IE72 Δ SUMO is a more potent transcriptional activator. Potential mechanisms underlying this difference in transactivation potential are being addressed, including alterations in IE72 stability or in its capacity to bind to other transcription factors.

The phosphopeptide sample which Dr. Spengler prepared by tryptic digestion of the phosphorylated PML protein followed by 2- dimensional thin layer chromatography has been analyzed by two types of mass spectrometry. Since the sample was of a very limited size, nanospray ionization was originally used. This technique consumes only 1 – 2 μ L per analysis and gives masses of all components present in the sample mixture without prior separation by HPLC. Two separate preparations from Dr. Spengler were provided and both produced similar spectra. The major peaks were selected for MS/MS whereby the spectrometer is set to isolate the selected ion, fragment it, and scan the fragment masses. Since peptides fragment preferentially at the peptide bonds, it is sometimes possible to deduce the amino acid sequence of a peptide from the masses of its fragments. Additionally, MS/MS experiments will produce an ion of $m/z=97$ ($H_2PO_4^-$) from phospho-S, T, or Y.

Unfortunately none of the peptide masses detected in the samples correspond to peptides ideally produced from tryptic digestion of the PML protein. However, there is clearly a phosphoserine – proline sequence present in the peptide. Other fragments detected suggest the sequence S_pPS_p or $S_pP(AP)/(PA)$; these possibilities all have the same mass. There are places in the sequence where these residues occur, but without identifying a few more residues or the “parent” mass of the tryptic peptide, it is difficult to identify the sample definitively.

I also sent a small portion of the remaining sample to Cornell for analysis by MALDI – MS. This technique is not generally useful for finding low molecular weight compounds (<1000 Da) but if a peptide were present in the higher mass range (as expected for a tryptic peptide) it may be possible to see by MALDI even though it was not detected on our instrument. Two very weak peaks were detected at m/z 1810.2 and 1881.9 but their intensity was not sufficient to obtain any fragment data.

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CHAPTERS IN BOOKS:

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SELECTED ABSTRACTS:

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DEPARTMENT OF THE ARMY

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLIS M. RINEHART
Deputy Chief of Staff for
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