Viral control of the SUMO pathway: Gam1, a model system
S. Chiocca
Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

Abstract
SUMO (small ubiquitin-related modifier) is a ubiquitin-like family member that is conjugated to its substrates through discrete enzymatic steps: activation, involving the E1 enzyme [SAE (SUMO-activating enzyme) 1–SAE2], conjugation, involving the E2 enzyme [Ubc9 (ubiquitin-conjugating enzyme 9)], and substrate modification, through the co-operation of Ubc9 and E3 protein ligases. Work from our laboratory has shown the first example of a viral protein, Gam1, that binds to the E1 heterodimer, inhibiting its function and causing a complete block of the SUMOylation pathway both in vivo and in vitro, followed by SAE1–SAE2 degradation. The mechanism by which a viral protein inactivates and subsequently degrades an essential cellular enzyme, arresting a key regulatory pathway, will be discussed. Although four distinct SUMO isoforms have been described, I will use SUMO to describe the entire system.

Gam1: a tool to study viral interference of cellular pathways
Viruses can be marvellous tools to study cellular pathways, since they have evolved many mechanisms to overcome cellular defences. Viral proteins are able to alter the host’s metabolic and replication functions in order to obtain an environment more favourable for viral proliferation. Viral proteins were among the first substrates found post-translationally modified by SUMO (small ubiquitin-related modifier) and their number is constantly increasing (reviewed in [1]). SUMOylation, the process of covalently attaching the SUMO moiety to a target protein, is a post-translational modification system. SUMO is covalently attached to its targets with four enzymatic reactions: SUMO proteins are first post-translationally processed by SUMO proteases to expose a C-terminal diglycine motif, which can then form a thioester bond with the catalytic cysteine residue of the E1 activating enzyme, the SAE (SUMO-activating enzyme) 1–SAE2 heterodimer. This activation step is followed by conjugation of the activated SUMO to the E2 conjugating enzyme Ubc9 (ubiquitin-conjugating enzyme 9) through the formation of a yet another thioester bond. In the final step, an isopeptide bond is formed between SUMO proteins and substrates through the mutual action of Ubc9 and E3 protein ligases. SUMO conjugation can be reverted by specific isopeptidases that hydrolyse the isopeptide bond, causing the removal of SUMO from its substrates (reviewed in [2]). SUMOylation is dynamic and reversible and is significantly dependent on these specific SUMO proteases both for processing newly translated SUMO to its active form during SUMO activation step and for de-SUMOylating substrates. The functional consequences of this modification are many and SUMOylation has been shown to increase protein stability, to alter protein–protein interactions to modify the subcellular localization of target proteins, to regulate transcription and to compete with other lysine-based modifications (reviewed in [2]). For viruses, the relationship between SUMOylation and viral proteins can be divided into two scenarios: viruses that have their proteins SUMOylated and viruses that have proteins that modify directly host SUMOylation, either by preventing de novo SUMOylation or by enhancing de-SUMOylation.
In both cases, the outcome should be a cellular environment more favourable for viral reproduction. Currently, the known SUMOylated viral proteins belong to both DNA and RNA viral families (reviewed in [1]). Less is known about the effects of viral infection on host SUMOylation. We have shown the first example of a viral protein, Gam1, that induces the disappearance of SUMO E1 and E2 and alters the SUMOylation status of host cell protein [3].

The adenovirus Gam1 was identified in a screen set-up to identify unknown viral pro-survivor proteins [4]. We immediately understood the importance of this protein for the virus, because a Gam1-negative CELO (chicken embryo lethal orphan) virus was replication-defective [5]. CELO is an avian adenovirus and its complete sequence revealed common adenoviral features, and also novel sequences rich in ORFs (open reading frames), among which is Gam1 [6]. It is possible that these ORFs replace the missing E1, E3 and perhaps E4 regions.

Gam1 and SUMO
The fact that Gam1 is essential for viral replication [5], and is a global activator of transcription and an inhibitor of HDAC (histone deacetylase) function [7], led us to consider the PML (promyelocytic leukaemia protein) NBs (nuclear bodies) [8]. The PML NBs are subnuclear structures made up of many...
different proteins, some of which are SUMO-modified. They are complex structures with no defined role yet, but one of their functions seems to be in antiviral defence. In fact, a number of viruses express proteins that disperse PML bodies (reviewed in [9,10]). Furthermore, the most reported effect of viruses on host cell SUMOylation concerns the PML NBs. The connection to SUMO came from our observation that concurrently with PML dispersion upon Gam1 expression, there was a clear and strong loss of SUMO-1 from the nucleus [8,11] and our results clearly demonstrated that Gam1 did not influence SUMO protease activity in vivo or in vitro [3]. We investigated how Gam1 blocked SUMOylation by carrying out a series of in vitro experiments, using the well-characterized SUMO substrate RanGAP1 (Ran GTPase-activating protein 1) [12]. The inhibition of the in vitro reaction occurred in a dose-dependent manner and only in the absence of an active SUMO–E1 intermediate [3]. Furthermore, a previously characterized mutant form of Gam1 (Gam1 LL/AA) [4,7,8] was also inactive in this assay [3]. Our results also showed that Gam1 could not compete with SUMO for E1 binding, but rather that it could directly inhibit E1 [3]. The most compelling result was the disappearance of SAE1–SAE2 (E1) and Ubc9 (E2) in vivo, both upon overexpressing Gam1 and during viral (CELO) infection, in agreement with our finding that Gam1 could also reduce the half-lives of both E1 and E2 [3].

Gam1, SUMO and ubiquitin

The induction of SAE1, SAE2 and Ubc9 disappearance by Gam1 could be prevented by the addition of the proteasome inhibitor MG-132 (the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal) [3], implicating the ubiquitin degradation pathway in Gam1 function. The dynamic and reversible post-translational process that covalently transfers ubiquitin to itself or to other proteins is called the ubiquitination pathway. Ubiquitin transfer requires different chemical steps catalysed by the sequential activity of different enzymes present both in the cytoplasm and the nucleus (reviewed in [13]). These enzymes are the single E1 activating enzyme, the E2 conjugating enzymes, the E3 ligases and, finally, the ubiquitin-specific proteases [DUBs (de-ubiquitinating enzymes)]. The ubiquitin E3s perform the rate-limiting selectivity step of direct substrate recognition and are the most numerous and diverse in the ubiquitination pathway. Many viral and bacterial pathogens exploit the cellular multisubunit ubiquitin ligases (E3s) to subvert normal cellular processes by degrading key endogenous proteins [reviewed in (14)]. The many ubiquitin E3s can be subdivided into different families based on their structural motifs. These include the CRLs [cullin RING (really interesting new gene) ligases].

We therefore carried out a bioinformatics analysis to identify any conserved domain or motif in the Gam1 amino acid sequence that might be traced back to any known ubiquitin ligase complexes and we found a putative SOCS (suppressor of cytokine signalling) motif in the C-terminus of the Gam1 amino acid sequence [15]. This domain and its N-terminal shorter motif, called a BC-box, are necessary for the interaction with the specific linker proteins EloB (elongin B) and EloC and are commonly found in proteins implicated in Cul2- and Cul5-based E3 ubiquitin ligase complexes (reviewed in [14]). Examining the alignment between the amino acid sequence of Gam1 and established SOCS motifs of known proteins, we observed that Gam1 has a conserved pattern of hydrophobic amino acids mainly in the first part of the SOCS domain, the BC-box [16]. The major differences between the SOCS-box motif in Gam1 and the SOCS-box consensus are the absence of the C-terminal P/L-rich region [17] and the lack of the highly conserved cysteine residue at position 6, where it is replaced by an alanine residue. The superimposition of the predicted α-helix of Gam1, amino acids 251–265, on to the three-dimensional structure of VHL (von Hippel–Lindau) helix 1 complexed with EloB–EloC [18] suggested that the putative BC-box of Gam1 could be functional. Applying this method, the hydrophobic residues Leu-252, Trp-255, Leu-258 and Leu-265 of Gam1 are located on the same face of the α-helix. The resulting steric surface and the relative position of these amino acids could support a successful interaction with the hydrophobic pocket of EloC. Confirming our working hypothesis, Gam1 was able to interact directly with EloC but not with EloB that is recruited to Gam1 only in the presence of EloC [16]. Subsequently, in agreement with the architecture of these ubiquitin E3 ligase complexes, in vivo we showed that Gam1 bound specifically and strongly to EloB, EloC and the RING protein Roc1 and simultaneously to Cul2 and Cul5 [16]. This result is quite surprising, since the cellular and viral proteins that interact with EloB and EloC usually form a complex in an exclusive manner with either Cul2 or Cul5, the only exception being the cellular protein VHL [19]. This cullin’s specificity seems to be due to the C-terminal P/L-rich region of the SOCS motif [17], which is absent from the Gam1 amino acid sequence. However, we could confirm the individual interactions between Gam1 and Cul2 or Cul5 and showed that the two cullins generate distinct complexes with the viral protein [16]. Through a series of mutants, we also identified the critical residues in the Gam1 SOCS motif required for association with Elo–cullin complexes. In fact, the double substitutions L258A and L265A (LL/AA) impair totally the Gam1 binding capability both in vitro and in vivo. Interestingly, this double point mutant was initially identified as the inactive version of Gam1 protein that failed in many phenotypes induced by this viral protein [3,4,7,8,11,20]. We therefore predicted that by recruiting cullin E3 ligase complexes through its BC-box, Gam1 could allow the specific and non-physiological degradation of specific cellular proteins to carry out its functions. To support this hypothesis, we decided to characterize in more detail the Gam1 complex in vivo. We speculated that Gam1, by recruiting the cullin E3 ligase complex through its BC-box, allowed SAE1 and SAE2 ubiquitination and their proteasomal degradation. Following this working model, we demonstrated that SAE1 and SAE2, but not Ubc9, were stably associated through the direct
link of Gam1 wild-type into the Cul2–Cul5–EloB–EloC–Roc1 complexes. The poor capacity of Gam1 mutant (Gam1 LL/AA) to interact with SAE1–SAE2 in vivo [3] is a direct effect of its failure to assemble in a stable manner the E3 ubiquitin ligase complex. To correlate the in vivo degradation of SUMO E1 and E2 and the assembly of E3 ubiquitin ligase complex induced by Gam1, we followed the enzymatic activity of these non-physiological protein aggregates. Unexpectedly, we showed that Gam1 could in vitro conjugate ubiquitin only on SAE1 protein, suggesting that the in vivo phenotype could be due to overlapping but distinct events. To explain the paradox that degradation of SAE1, SAE2 and Ubc9 in vivo is mediated by Gam1 while being unable to post-translationally modify SAE2 and Ubc9 in vitro, we followed the possibility that SUMO enzymes are mutually stabilized in vivo. Using an RNA interference approach, we partially validated this hypothesis, demonstrating that the absence of any of the SUMO E1 components induces the degradation of the other subunit [16] but did not affect the stability of Ubc9 under our conditions. Therefore we could conclude that the stability of the SUMO E1 heterodimer is closely related to the presence of its two subunits, SAE1 and SAE2, and that the effect of Gam1 on SAE2 seems to be a consequence of the induced ubiquitination and degradation of SAE1 [16]. Unfortunately, the mechanism of Ubc9 degradation remains to be understood, but is, nevertheless, strictly linked to the function of Gam1 and independent of SUMO E1 degradation. It is interesting to underline that SAE1 targeting by Gam1 reflects the pivotal role of this protein in the regulation of SUMOylation.

In conclusion, in our model, the adenoviral protein Gam1 binds directly to the SAE1–SAE2 heterodimer, inducing its enzymatic inhibition. Through its BC-box motif, Gam1 recruits the adaptor heterodimer EloB–EloC and the structural proteins Cul2–Cul5 to assemble functional ubiquitin E3 ligase complexes. The correct formation of this cullin-based complex could stabilize the binding between Gam1 and SAE1–SAE2. The cullins, involved in these complexes, recruit the RING protein Roc1 to allow SAE1 ubiquitination through the ubiquitin–E2 enzyme. The consequent proteasomal degradation of SAE1 destabilizes SAE2 and facilitates its ubiquitination and degradation.

Moreover, Gam1 causes the degradation of Ubc9 by an as yet uncharacterized pathway.

I thank all past and present members of my laboratory, the European Institute of Oncology and IEO-IFOM [FIRC (Fondazione Italiana Ricerca Cancro) Institute for Molecular Oncology] campus. I also thank Associazione Italiana per la Ricerca sul Cancro and the Italian Ministry of Health for financial support.

References

Received 9 July 2007 doi:10.1042/BSI0351419