Comparison of the SUMO1 and ubiquitin conjugation pathways during the inhibition of proteasome activity with evidence of SUMO1 recycling

Daniel BAILEY and Peter O'HARE¹

Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, U.K.

To investigate potential interplay between the SUMO1 (small ubiquitin-related modifier-1) and ubiquitin pathways of post-translational protein modification, we examined aspects of their localization and conjugation status during proteasome inhibition. Our results indicate that these pathways converge upon the discrete sub-nuclear domains known as PML (promyelocytic leukaemia protein) NBs (nuclear bodies). Proteasome inhibition generated an increased number of PML bodies, without any obvious increase in size. Using a cell line that constitutively expresses an epitope-tagged version of SUMO1, which was incorporated into high-molecular-mass conjugates, we observed SUMO1 accumulating in clusters around a subset of the NBs. Nuclear ubiquitin was initially observed in numerous speckles and foci, which bore no relationship to PML NBs in the absence of proteasome inhibition. However, during proteasome inhibition, total ubiquitin-conjugated species increased in the cell, as judged by Western blotting. Concomitantly the number of nuclear ubiquitin

clusters decreased, and were almost quantitatively associated with the PML NBs, co-localizing with the SUMO-conjugated pool. Proteasome inhibition depleted the pool of free SUMO1 in the cell. Reversal of proteasome inhibition in the presence or absence of protein synthesis demonstrated that free SUMO1 was regenerated from the conjugated pool. The results indicate that a significant fraction of the free SUMO1 pool could be accounted for by recycling from the conjugated pool and indeed it may be that, as for ubiquitin, SUMO1 needs to be removed from conjugated species prior to processing by the proteasome. Taken together with other recent reports on the proteasome and PML NBs, these results suggest that the PML NBs may play an important role in integrating these pathways.

Key words: deconjugation, proteasome, promyelocytic leukaemia protein (PML), small ubiquitin-related modifier (SUMO1), ubiquitin.

INTRODUCTION

Post-translational modification of proteins by ubiquitin is a key mechanism for regulating cellular processes (reviewed in [1,2]). In addition to its central role in the degradation of proteins, ubiquitination performs diverse roles in protein trafficking and regulates a number of crucial cellular functions. Similarly SUMO1 (small ubiquitin-related modifier-1) (also termed sentrin [3], PIC1 [4], or GMP-1 [5,6]) has been shown, in many recent studies, to influence the activities of a range of proteins, principally transcription factors (for reviews see [7,8]). A number of other ubiquitin-like post-translational modifications have been identified; though less well understood, they are also gaining recognition for their involvement in regulating protein localization, stability or function (reviewed in [9]).

There are similarities in the pathways involved in activating and conjugating ubiquitin and ubiquitin-like proteins to particular lysine residues within target proteins. However, distinct differences occur, providing scope for individual regulatory mechanisms. The reversal of these post-translational modifications may also occur through similar processes, but with differences in the utilization and regulation of the specific proteases that cleave the conjugated moieties from the various targets. Many details of the general turnover, processing and inter-relationships between the pathways remain poorly understood.

Ubiquitination is the key cellular modification involved in processing proteins for degradation via the proteasome pathway and regulating protein stability. However, SUMO1 modification has also been implicated in regulating protein stability and in

some cases SUMO1 has been demonstrated to be antagonistic to ubiquitination [10,11]. For example, the transcription-regulating protein, I κ B α (inhibitory κ B), is protected from signal-induced degradation by the occupation of the targeted lysine moiety by SUMO1 [10]. Studies of the RING finger protein, MDM2 (murine double minute clone 2 oncoprotein), have demonstrated that this protein functions as a ubiquitin E3 ligase (which may be a general feature of the RING finger proteins), both for itself and for p53 [12]. SUMO1 modification of MDM2 appears to protect the protein from self-degradation, whilst promoting the degradation of p53 [13]. However, the mechanism of exchange between SUMO1 and ubiquitination remains unclear.

A potential connection between the ubiquitin and SUMO1 pathways arises from studies on the PML (promyelocytic leukaemia protein) protein. PML is also a member of the RING finger family of proteins [14]. It is a major constituent of the PML NBs (nuclear bodies), discrete domains within the nucleus that are enriched in a variety of proteins. Their exact cellular function has been the subject of much speculation, including proposed roles in transcriptional regulation, apoptosis and genomic stability (reviewed in [15–18]). PML was one of the first demonstrated targets of SUMO1 modification. A variety of other SUMO1-modified proteins have also been reported to localize to NBs [including nuclear antigen Sp100, WRN (Werner syndrome) and p53]. It has been suggested that SUMO1 modification, at least of PML, may be important for recruiting PML to the NB structure [19,20] and also for the recruitment of PML interacting proteins such as Daxx (death-associated protein 6) [21].

Abbreviations used: GFP, green fluorescent protein; HA, haemagglutinin; HA-SUMO-NC, non-conjugatable HA-tagged SUMO1; hmSUMO1, Myc-epitope-tagged SUMO1; MDM2, murine double minute clone 2 oncoprotein; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; NBs, newborn calf serum; NB, nuclear body; PML, promyelocytic leukaemia protein; PSI, proteasome inhibitor I; SENP1, SUMO1/sentrin-specific peptidase 1; SUMO, small ubiquitin-related modifier; USP7, ubiquitin-specific protease 7 (herpes virus-associated).

¹ To whom correspondence should be addressed (email P.O'Hare@mcri.ac.uk).

With regards to the ubiquitin pathway, it has previously been suggested that misfolded proteins may be deposited at the PML NB [22]. Moreover a ubiquitin-specific protease known as USP7 [ubiquitin-specific protease 7 (herpes virus-associated)], also known as HAUSP] has been reported to localize to NBs [23], as have components of the proteasome itself [24,25]. Furthermore arsenic trioxide treatment is known to increase the accumulation of SUMO1-modified PML species [19], promote proteasome recruitment to the PML body and increase the degradation of PML [26]. These and other data raise interesting questions on the relationship between ubiquitin and ubiquitin-like protein modifications. For example, the proteasome is normally considered to unfold proteins and feed them into its barrel structure to degrade the protein, but it remains unclear how the branched structures of ubiquitin-like proteins conjugated to target molecules are degraded. Recent work by Yao and Cohen [27] demonstrated the existence of a proteolytic activity within the 19 S subunit (that forms the proteasome lid) that was necessary for cleaving the ubiquitin side chain to permit ubiquitin recycling. It is not clear in the case of ubiquitin-like molecules whether there is also a requirement for pre-cleavage to enable the recycling of the conjugated ubiquitin-like molecule or whether the branch-like molecules can be processed and degraded without cleavage. If side chain removal is required for SUMO and other ubiquitin-like modifiers, it is currently unclear whether the same proteolytic activities as those for removing ubiquitin chains are involved. Much remains to be determined on how the processes, dynamics and site(s) of modification, deconjugation or degradation are regulated.

In this paper, we examine the localization and regulation of components of the ubiquitin and SUMO1 modification pathways during proteasome inhibition. We also investigate whether SUMO1 deconjugation occurs prior to degradation of SUMO1-modified proteins.

Our results indicate that the SUMO1 and ubiquitination pathways converge upon PML NBs. In a cell line engineered to express an epitope-tagged SUMO1, prior to proteasome inhibition, a subset of SUMO1 (presumably that conjugated to PML and PML NB constituents) was found at the NBs. The remainder of SUMO1 (conjugated or free) showed little specific localization. Following proteasome inhibition, the majority of SUMO1 was found to coalesce around a subset of the NBs, with abundant SUMO1 species, separate from the PML-conjugated species emanating from the bodies. Nuclear ubiquitin was initially observed in numerous speckles and foci unrelated to PML NBs. However, during proteasome inhibition, while total ubiquitin-conjugates increased in the cell, the number of nuclear ubiquitin clusters decreased and conjugated ubiquitin species accumulated at the PML NBs. Reversal of proteasome inhibition, in the presence or absence of *de novo* protein synthesis, demonstrated that free SUMO1 was regenerated from the conjugated pool simultaneously with protein degradation. Indeed a significant fraction of the free SUMO1 pool could be accounted for by recycling from the conjugated pool. It may be that, as for ubiquitin, SUMO1 must be removed from conjugated species prior to processing by the proteasome. The results, together with recent additional evidence, are discussed with reference to the proposal that PML NBs may play an important role in integrating SUMO and ubiquitin pathways.

EXPERIMENTAL

Cells and DNA constructs

Hep2 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum and penicillin and

streptomycin at 100 units/ml and 100 µg/ml respectively. Hep2-SUMO cell lines have been described previously [28] and were cultured under similar conditions with the addition of 2 µg/ml puromycin to maintain the integrated SUMO1. The myc-tagged SUMO1 construct has been previously described. [28]. HA (haemagglutinin) epitope-tagged SUMO1 constructs were constructed using PCR and cloned into a pcDNA3 backbone. The HA-SUMO-NC (non-conjugatable) construct was produced by using PCR mutagenesis to introduce a glycine to histidine substitution at the second glycine residue normally utilized as a donor for conjugation, followed by a stop codon.

Transfections

Transfections were performed using the calcium phosphate precipitation procedure modified by the use of Bes-buffered saline (pH 7.06) as previously described [29]. The total amount of DNA was equalized to 2 µg with pUC19 DNA.

Immunofluorescence studies

Cells were plated on glass coverslips placed in plastic tissue culture vessels at 1×10^5 cells/35 mm well. Approximately 40 h post-transfection, cells were washed in PBS, and fixed with ice-cold methanol. Primary antibodies were diluted in PBS/10% (v/v) newborn calf serum (NBCS), and applied for 20 min. Primary antibodies used were anti-c-myc 9E10 (1:400, Boehringer Mannheim) for the myc-tag, anti-GMP-1 (1:1000, Invitrogen) or anti-PIC1 (1:200, kindly supplied by P. Freemont) for SUMO1. A rabbit polyclonal antibody to PML (DB#75) was generated against a purified GST (glutathione S-transferase)-PML bacterial expression product. Specificity of the antibody was verified by comparisons with the existing anti-PML monoclonal antibody (5E10 [29a]). Fluorochrome-conjugated secondary antibodies of appropriate specificity, goat anti-mouse or goat anti-rabbit, conjugated to Alexa 488 or Alexa 543 dyes were obtained from Molecular Probes. The fluorochrome-conjugated secondary antibodies were diluted 1:200 in PBS/10% (v/v) NBCS and applied to the coverslip. Following washing, cells were visualized using a Zeiss LSM 410 confocal microscope imaging system. Images for each channel were captured sequentially with 8-fold averaging at an image size of 512×512 pixels. Composite illustrations were prepared using Adobe software. Example images shown are representative of numerous images gathered for each test construct and condition.

Western blot analysis

Proteins from cell extracts were analysed by separation either on SDS/10% polyacrylamide gels prepared and run in the Bio-Rad Mini-Protein II apparatus, or with commercially prepared gel systems (Invitrogen). The proteins were transferred to nitrocellulose membranes, which were then blocked with PBST [PBS/0.05% (v/v) Tween 20] containing 5% (w/v) non-fat dried milk. After blocking, membranes were incubated with primary antibody in PBST/5% (w/v) dried milk for 1 h, washed three times in PBS/1% (v/v) Triton X-100 and incubated for a further 1 h with PBST/5% (w/v) dried milk containing the appropriate horseradish peroxidase-conjugated secondary antibody. Following further washing in PBS/1% (v/v) Triton X-100, membranes were processed using chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL, U.S.A.). Primary antibodies used for immunoblotting were anti-actin AC-40 (1:500, Sigma), anti-c-myc 9E10 (1:400, Boehringer Mannheim) and anti-ubiquitin FK2 (1:10000, Biomol International).

Figure 1 Effects of proteasome inhibition by MG132 on endogenous PML and SUMO1

Hep2 cells were treated for 7 h with either DMSO (control) or 10 μ M MG132. Cells were subsequently fixed with methanol and immunofluorescence was performed to detect endogenous PML (a and d) using an anti-PML polyclonal antibody (DB#75) and endogenous SUMO1 (b and e) using anti-PIC1 antibody. Corresponding merged panels are shown (c and f); arrows indicate examples of co-localization of SUMO1 and PML at the NBs.

Proteasome inhibition studies

Proteasome inhibitors MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), lactacystin and PSI (proteasome inhibitor I) were obtained from Calbiochem. Inhibitors were dissolved in DMSO and applied to cells for the time periods and at the concentrations indicated in the text. An equivalent volume of DMSO was added to control cells (not exceeding 0.1 % DMSO).

RESULTS

Analysis of endogenous SUMO1 and PML at the NB following proteasome inhibition in Hep2 cells

We wished to examine the relationship between the ubiquitin–proteasome pathway, NBs and SUMO1 modification. To this end we examined the effect of the reversible proteasome inhibitor, MG132, on the localization of SUMO1 and PML NBs in Hep2 cells. MG132 induced a pronounced increase in the number of PML NBs from approximately 3–5 to 8–15 per cell (Figures 1a and 1d). Although there was an increase in the number, the NBs still retained a generally similar maximum size although sometimes exhibiting an altered, less regular morphology (Figures 1a and 1d short arrowhead). Since NBs have been reported to be maintained by covalent linkage of SUMO1 to PML [30], we next examined the status of endogenous SUMO1 within these cells by co-staining for SUMO1. The results showed that the increase in PML-containing NBs was also reflected by an increase in SUMO1 recruitment into the additional bodies (Figures 1b and 1e).

PML is a major component of the NBs and SUMO1 modification of PML has been reported to target PML to NBs. The increased accumulation of SUMO1 at new NBs could be solely due to an increase in the SUMO1 modified isoforms of PML, due to an increase in additional protein species modified by SUMO1 or potentially also to an increase in free SUMO1.

Analysis of SUMO1 and PML NBs in a cell line expressing epitope-tagged SUMO1

Generally, however, the relatively low levels of endogenous SUMO1 limited our attempts to examine changes in SUMO1 levels and SUMO1 conjugation by biochemical analysis. To facilitate further investigation of SUMO1 conjugation during proteasome inhibition, we used established cell lines that constitutively express hmSUMO1 (myc-epitope-tagged SUMO1) [28]. In these cells hmSUMO1 was present in predominantly diffuse nuclear staining, with frequent accumulation in foci often co-localizing with PML (Figure 2, arrowed). We have not observed SUMO1 conjugation to proteins lacking the SUMO1 consensus sequence within this cell line. However, as with the establishment of any cell line for the expression of a target protein, we cannot rule out any effects of exogenous expression as such. Nonetheless, we note that we have also been unable to detect SUMO1 conjugation to a PML mutant that lacks two key lysine substrates for SUMO-modification, whilst retaining numerous other lysines. These observations indicate that the SUMO1 conjugates that we observe within our stable cell line are derived from the specific covalent linkage of SUMO1 to appropriate target proteins.

We have previously reported [28] that the introduced hmSUMO1 acted as a substrate for conjugation into high-molecular-mass conjugates. We also reported that the majority of hmSUMO1 within these cell lines appeared to be conjugated in high-molecular-mass species, with comparatively minor amounts of free SUMO1 (see also Figure 4).

We next examined the effect of MG132 treatment on localization of PML and SUMO1 in these cells. Within 1 h of proteasome inhibitor treatment there was little change in the PML bodies or SUMO1 distribution (Figure 3a). However, as indicated above, by 3 h a significant increase in the number of PML-containing NBs had occurred. SUMO1 also accumulated within these bodies

Figure 2 Localization of SUMO1 in a cell line constitutively expressing epitope-tagged SUMO1

A Hep2-SUMO cell line expressing a myc epitope-tagged SUMO1 protein (hmSUMO1) was fixed and immunofluorescence staining was performed to detect hmSUMO1, using an anti-myc antibody (9E10), or with a polyclonal antibody to detect PML. Expression of hmSUMO1 was mainly nuclear diffuse, although some dense staining nuclear domains were also observed. The arrows in the separate images indicate dense staining areas, enriched for SUMO1 that co-localized with PML (see merge image).

(Figure 3a, arrowed) with separate channels showing clear co-localization of the tagged SUMO1 with the induced PML bodies. By 7 h post-treatment there was no further increase in the size or number of PML foci. However, SUMO1 clearly continued to accumulate at the PML NBs. The majority of the total SUMO1 population could now be seen in foci frequently surrounding and extending well beyond the PML bodies which, although increased in number, retained their original size (Figure 3A, 7 h and 7 h high magnification, arrows). Similar results, showing an increase in the number of PML-containing NBs and the profound accumulation of SUMO1 into and around these bodies, were obtained with two additional proteasome inhibitors, PSI and lactacystin (Figure 3b, arrowed).

Together, these results show that in the absence of proteasome inhibition, the majority of hmSUMO1 was incorporated into high-molecular-mass conjugates with no obvious selective localization, apart from the population present in pre-existing PML NBs. However, upon proteasome treatment and with the resultant increase in NBs, virtually all the SUMO1 population could now be observed congregating in and emanating from the NBs. A qualitative examination of PML and SUMO1 localization indicated that all the major SUMO1 depositions were in association with PML NBs. However, there is no formal way to discriminate between the pre-existing and the MG132-induced NBs. Since from the Western blot analysis, the majority of hmSUMO1 was in the form of high-molecular-mass conjugates, particularly in the presence of MG132 (see below, Figure 6), the most likely explanation for these results is the accumulation of the SUMO1 conjugates other than PML at and around the induced NBs.

To further characterize the accumulation of SUMO1 at the NB sites, we transfected the hmSUMO1 cell line with a separately tagged construct (HA) derived from a non-conjugatable SUMO1 (HA-SUMO-NC). This variant contains an amino acid substitution at the C-terminal glycine and was terminated after this position, so as to mimic the processed deconjugated SUMO1, whilst also preventing it being conjugated by the SUMO-conjugation pathway. Examination of the non-conjugatable SUMO1 by Western blot demonstrated that it was only present as a single species within the cell, confirming that it was neither utilized for conjugation, nor itself a target for SUMO1 conjugation into chains (Figure 4a; compare lane 2 versus lane 3).

In parallel with the normal HA-SUMO1 construct as a control, we examined the localization of the HA-SUMO-NC variant in both the presence and absence of proteasome inhibition. In this experiment, the endogenous SUMO1 in the cell line is detected by the myc-tag and anti-myc antibody, while the transfected HA-

SUMO1 or HA-SUMO-NC is detected by the HA-tag and corresponding antibody. In the absence of proteasome inhibition HA-SUMO1 accumulated in the nucleus, localizing with the normal SUMO1 as shown above (Figure 4b, control; compare panels a and b, short arrows). In contrast, the non-conjugatable HA-SUMO-NC showed a diffuse profile throughout the cell and was not recruited to the SUMO1 domains (Figure 4b, control; compare panels d and e, long arrows). Furthermore, upon proteasome treatment HA-SUMO1 was recruited and co-localized with the myc-tagged SUMO1 domains seen above (Figure 4b, MG132 treated; compare panels g and h, short arrows). In contrast the HA-SUMO-NC variant remained diffuse throughout the nucleus and cytoplasm (Figure 4b, MG132 treated; compare panels j and k, long arrows). Expression of the non-conjugatable SUMO1 alone did not appear to interfere with, or prevent the accumulation of, other SUMO1 conjugates at the PML NBs (Figure 4B, panel k). These results suggest that the normal selective partitioning of SUMO1, into the nucleus, is maintained by interaction of SUMO1 with either its target substrates or components of the conjugation pathway. The results also confirm that the accumulation of SUMO1, in the presence of MG132, is due to the conjugated SUMO1 protein complexes accumulating at the PML NB.

Localization of SUMO1 in relation to conjugated ubiquitin

Recent reports have linked PML NBs to the proteasome-dependent pathway of proteolysis [24,26,31], although the precise nature of the association is unclear. Our observations of increased NB formation led us to examine the general association between PML, SUMO1 and ubiquitin localization during proteasome inhibition. Hep2-SUMO cells were treated with proteasome inhibitor and the localization of ubiquitin was examined using a monoclonal antibody that recognizes the conjugated forms of ubiquitin [32]. In the absence of proteasome inhibitors, ubiquitin was localized in many speckled domains upon a diffuse background, mainly within the nucleus. (For these experiments SUMO1 detection was performed using a polyclonal anti-SUMO1 antibody, rather than the monoclonal antibody to the epitope tag.) These ubiquitin foci and speckles were much more numerous than the PML NBs and there was no significant co-localization between the two types of structures (Figure 5a, left hand panel, arrows). However, upon proteasome inhibition ubiquitin localization altered significantly. The ubiquitin domains decreased in number and were now seen to re-localize to the PML domains, with large ubiquitin-containing clusters frequently surrounding the PML domains (Figure 5a, right hand panel, arrows). Moreover,

Figure 3 Effect of proteasome inhibition (MG132) on PML and exogenous SUMO1

(a) Hep2-SUMO cells were treated for up to 7 h with DMSO (Control) or 10 μ M MG132. Cells were subsequently fixed with methanol and immunofluorescence was performed to detect epitope-tagged SUMO1 (red) using anti-myc antibody, and endogenous PML (green) using an anti-PML polyclonal antibody (#75). Arrows indicate representative NBs showing recruitment of hmSUMO1. (b) Similar results were observed with Hep2-SUMO cells treated for 7 h with either 20 μ M PSI or 50 μ M lactacystin and processed in a similar manner. MG132-treated and control cells are shown for comparative purposes.

the ubiquitin distribution under these conditions was similar in appearance to the SUMO1 localization that we had observed earlier. We therefore wished to check for any co-localization between the re-localized ubiquitin and SUMO1 (Figure 5b). In the absence of MG132, ubiquitin exhibited little significant co-localization with SUMO1 (Figure 5b, minus MG132). However,

upon proteasome inhibition, the two proteins were observed in prominent accumulations within the nucleus, with strong co-localization between the two (Figure 5b, plus MG132, short arrows). The co-localization, taken together with the previous localization of SUMO1 clusters at the PML body (Figure 3) and the corresponding ubiquitin clusters at the PML

Figure 4 Analysis of a non-conjugatable variant of SUMO1

(a) Hep2-SUMO cells were separately transfected with mock, wild-type HA-SUMO1 or the HA-SUMO NC variant. Cells were subsequently harvested by cell lysis, extracts separated by electrophoresis, and immunoblotting performed. Separations were probed for HA-SUMO1 using an antibody to the HA-epitope tag. (b) Hep2-SUMO-NC cells were separately transfected with wild-type HA-SUMO1 (a–c and g–i) or (HA-SUMO-NC (d–f and j–l). Cells were treated with DMSO (control) or 10 μ M MG132 for 5 h, prior to fixation and staining with anti-HA antibody for the transfected SUMO1, or with antibody 9E10 for the integrated myc-tagged SUMO1. The wild-type HA-SUMO localized with the standard SUMO1 profile (a versus b), whereas the HA-SUMO-NC variant was diffuse (d versus e) throughout the cytoplasm and nucleus. Upon MG132 treatment the wild-type HA-SUMO was recruited to SUMO1 domains (g versus h, short arrows), whereas the HA-SUMO-NC remained diffuse and was not recruited to SUMO1 domains (j versus k, long arrows). Respective merge images are shown in c, f, i and l.

body, suggested that these two post-translational modifications were converging on the same subcellular location. We also observed similar co-localization, upon proteasome inhibition, between SUMO1 and ubiquitin, or PML and ubiquitin, for the parental Hep2 cells, albeit with smaller foci (results not shown). Interestingly, co-localization between ubiquitin and SUMO1 was observed only for the nuclear species. Although pronounced recruitment and accumulation of ubiquitin into punctate structures was also observed in the cytoplasm, no recruitment of SUMO1 was observed here (Figure 5b, plus MG132, long arrows).

To determine if the accumulation of the ubiquitinated and SUMO1-modified proteins around the PML body was a specific effect, we examined the localization of other normally diffuse nuclear proteins, such as CDK6 (cyclin-dependent kinase 6) and the transcription factor Sp1. These proteins did not accumulate at

the NB to any significant extent (results not shown), indicating that the accumulation of SUMO1 and ubiquitin was the result of a block at a particular point in a common regulatory or processing pathway for these modifications. Similarly, we did not observe any appreciable recruitment of proteasome subunits to these clusters (results not shown). Thus although ubiquitin domains and foci appeared generally unrelated to NBs in untreated cells, proteasome inhibition revealed a significant relationship. The reduction in ubiquitin foci is not simply due to a loss of conjugated species (indeed ubiquitin-conjugated species increased under these circumstances). Rather we believe that these results are best explained by a flux of ubiquitin-conjugated species through the NBs and, under circumstances where proteasome function is inhibited, such conjugates become retained or trapped in the NBs, resulting in expanded ubiquitin clusters emanating from them.

Figure 5 Localization of SUMO1 in relation to conjugated ubiquitin

Hep2-SUMO cells treated with DMSO (control) or 10 μ M MG132 for 7 h were fixed with methanol and immunofluorescence analysis was performed. **(a)** Conjugated ubiquitin was detected with anti-ubiquitin FK2 monoclonal antibody (green), and PML with anti-PML #75 polyclonal antibody (red). Arrows indicate PML domains. **(b)** Hep2-SUMO cells co-stained for conjugated ubiquitin with anti-ubiquitin FK2 (green) and SUMO1 with anti-PIC1 antibody (red). Short arrows indicate representative domains with SUMO1 and ubiquitin co-localization, whilst long arrows indicate accumulation of cytoplasmic ubiquitin.

Western blot analysis of SUMO1 modification and ubiquitination during MG132 treatment

Our results on the recruitment of SUMO1 to the NBs, and the corresponding co-localization with ubiquitin, prompted us to perform biochemical analyses of the general status of SUMO1 and ubiquitinated proteins following MG132 treatment. Hep2-SUMO cells were treated with MG132 for different intervals (up to 7 h). Total cell extracts were harvested at different time points and proteins separated by electrophoresis on SDS/PAGE gels. Samples were probed with an antibody either to conjugated ubiquitin or to the epitope-tagged SUMO1. For the purposes of detection of both conjugated and unconjugated SUMO1 species, proteins were analysed on 10–20 % (w/v) polyacrylamide gradient SDS gels, while detection of ubiquitin conjugated species was performed using 3–8 % gradient gels. The results confirmed that, as expected, MG132 dramatically increased the levels of conjugated ubiquitin. Within 1 h of treatment, relatively abundant high-molecular-mass ubiquitin-conjugated species had increased

dramatically, from a low background level (conjugated ubiquitin can be detected at this time point on longer exposures of the blots, results not shown). Thereafter there was additional slow accumulation until 7 h, the last time point studied (Figure 6, anti-ubiquitin). Inhibition of the proteasome also increased the levels of conjugated SUMO1 (Figure 6, anti-myc). With the increase in SUMO1-conjugated species, there was a concomitant decrease in unconjugated SUMO1 (Figure 6, anti-myc). The levels of actin, utilized as a marker for equivalent loading, remained relatively unchanged (Figure 6, anti-actin). The accumulation of SUMO1-conjugated species appeared to lag behind that of ubiquitin-conjugated species. Between 0 and 3 h, there was a significant increase in ubiquitin-conjugated species, but little effect on the total abundance of SUMO-conjugated species. However, this analysis does not take into account the potential differences in abundance and dynamics of ubiquitin turnover between the different cellular compartments. In particular, we have found in immunofluorescence studies that the cytoplasmic compartment exhibits a greater accumulation of conjugated ubiquitin compared

Figure 6 Western blot analysis of SUMO1 modification and ubiquitination during MG132 treatment

Hep2-SUMO cells treated with 10 μ M MG132 for 7 h were harvested by cell lysis. Cell extracts were separated by electrophoresis and immunoblotting performed. SDS/10–20%-PAGE gel separations were probed for hmSUMO1 using an anti-myc (9E10) antibody. SDS/3–8%-PAGE gel separations were probed for conjugated ubiquitin using anti-ubiquitin FK2. Equivalent samples were also probed with an anti-actin polyclonal antibody as a control for loading.

to the nucleus (Figure 5b, long arrows; also compare cytoplasmic ubiquitin, control versus MG132 treatment). Further comparisons of the ubiquitin and SUMO1 accumulations, by immunofluorescence, demonstrated that the rate of accumulation of nuclear ubiquitin conjugates was similar to that of the rate of SUMO1 conjugate accumulation (results not shown).

We next examined the effect of releasing the proteasome block on the accumulation of SUMO1-conjugated species. In this analysis, conditions were optimized to drive the accumulation of SUMO1 into conjugated species and deplete the pool of unconjugated species. After MG132 treatment, cells were then washed to remove the reversible proteasome inhibitor and incubation continued in either the absence or presence of cycloheximide to block new protein synthesis. The cells were then harvested at intervals and SUMO1 species examined as before (Figure 7a). Consistent with our earlier results, the level of SUMO1-conjugated species increased by 7 h of MG132 treatment and free SUMO1 was reduced to undetectable levels (Figure 7b, lanes 4 and 5). Upon reversal of MG132 inhibition, the high-molecular-mass SUMO1 conjugates were lost with time, with a detectable reduction within 1 h and complete reduction to starting levels by 3–6 h. A corresponding increase in free SUMO1 was observed, starting at approximately 1 h post-release and returning to basal levels (as seen in the absence of drug) by 6 h (Figure 7b, lane 11). The free SUMO1 pool also returned to virtually normal levels in the presence of an inhibitor of new protein synthesis (cycloheximide). This indicated that the increase in the free SUMO1 pool was derived largely from the conjugated SUMO1

pool, and was not solely due to new protein synthesis (Figure 7b, lane 12). The increase in free SUMO1 following MG132 removal suggests that SUMO1 deconjugation occurs during resumption of proteasome-mediated degradation. It is interesting to note, however, that the total accumulation of free SUMO1 did not appear to account for the total population of SUMO1 that was lost from the conjugated species upon reversal of proteasome inhibition. This could indicate that SUMO1-conjugated species, together with the SUMO1 moiety, are degraded by the proteasome. Alternatively deconjugated SUMO1 could, by itself, be degraded after removal from the targets. Although we cannot formally distinguish between these possibilities, we note that upon proteasome treatment we were unable to detect ubiquitin modification of the non-conjugatable SUMO1 (results not shown).

DISCUSSION

Detailed understanding of the SUMO1 modification pathways is hampered by the low levels of endogenous SUMO1, making it difficult to analyse biochemical and cell biological aspects of this pathway. Our results, using a cell line constitutively expressing a tagged version of SUMO1, have facilitated analysis of several aspects of SUMO1 modification and provide evidence for the convergence of the ubiquitination and SUMO1 modification pathways at the PML NB. We demonstrate that proteasome inhibition leads to increased numbers of PML NBs, an increase in the SUMO1-conjugated species in the cell and the accumulation of SUMO1 at NBs, which is consistent with earlier results [23]. The specific increase in number, but not size, of the NBs warrants comment, because the result implies that during proteasome inhibition, PML is not simply recruited to the pre-existing NBs but is recruited to newly formed NBs. These may themselves have some pre-existing substructure, which has not been detected, or are formed *de novo*. Without knowledge of any candidate constituent of the new NBs, it is difficult to make conclusions on which of these explanations is correct. However, it is clearly possible that the MG132-induced NBs have different constituents and potentially different roles during the response and release from inhibition. Consistent with this, dynamic differences in NB composition have been suggested from a number of studies [26,33,34]. Thus the accumulation of SUMO1 around the NBs could reflect accumulation at a distinct subset, but this is presently difficult to determine. This notwithstanding, since our biochemical analysis demonstrated that after proteasome inhibition, the majority of SUMO1 was in the form of multiple high-molecular-mass conjugates, the accumulation of SUMO1 at NBs was probably due not only to those proteins normally resident in NBs, but also to a wide variety of SUMO1-conjugated species. This conclusion is important since previously it has been difficult to determine whether the increased SUMO1 accumulation at NBs during proteasome inhibition is due to PML, the main constituent and main NB SUMO1-modified protein. Our analysis demonstrates clustering of SUMO1 conjugates emanating out from PML NBs and, clearly, not solely due to PML itself. The accumulation of SUMO-modified species is most likely due to inhibition of their degradation. But it could be for example that proteasome inhibition leads to an accumulation of an inhibitor of deSUMOylating enzymes. This notwithstanding, our observations indicate that the bulk of the cellular SUMO1-conjugated species traffic through NBs. Although speculative, it may be that this represents the normal route of processing for further modification or breakdown of the vast majority of SUMO1-conjugated proteins in the cell.

We note that although there appears to be no obvious association between NBs and nucleoli in the absence of proteasome inhibitors,

Figure 7 Effect of proteasome release on SUMO1 conjugation

(a) Schematic of the time course experiment. Hep2-SUMO cells treated with DMSO (control) or 20 μ M MG132 for 7 h were washed and media replaced $\pm$ cycloheximide at 100 μ g/ml. Cells were incubated for up to 6 h prior to harvesting, then separated by electrophoresis and immunoblotting performed. (b) Extracts were probed for SUMO1 (using an anti-myc antibody). Equivalent samples were also probed with an anti-actin polyclonal antibody as a control for loading.

it was recently reported that PML relocates from the NBs to the nucleolus upon treatment with proteasome inhibitors [25]. We did not observe significant localization of PML or SUMO1 to the nucleolus, even after prolonged MG132 treatment (results not shown) though we did observe some nucleolar localization in a minor population ($< 1\%$) when studying another cell type (Vero cells). Although we do not know the reason for the difference, it is interesting to note that PML was recently reported to move to the nucleolus upon DNA damage [35]. Although this was not the case for our studies of proteasome inhibition, it is conceivable that additional stresses were induced in other studies [25].

We observe that proteasome inhibition led not only to the accumulation of SUMO1 species at the NBs, but also to pronounced co-localization with ubiquitin-conjugated species. Our results provide support for the proposal that the PML NB may be part of the processing pathway for the regulation and sorting of proteins for degradation. Consistent with this, previous results have identified the ubiquitin-specific protease (USP7 or HAUSP) as a component of the PML domains [36]. USP7 has been implicated in the stabilization of p53 by deubiquitination [37]. Given the localization of USP7 to the NBs, it would seem reasonable to suggest that ubiquitinated p53 would localize to the PML NBs. SUMO1 modification of p53 could also affect or regulate this event, though this is presently unclear. Other evidence for an involvement of the proteasome pathway with NB function has been reported, whereby interferon treatment enhances PML body formation and increases the recruitment of proteasome activator PA28 to the nuclear body [24]. Viral antigens that are misfolded and destined for degradation have been reported to be deposited at the PML NB [22]. PML itself is a member of the RING finger

family of proteins and there is now robust evidence to suggest that the RING finger motif, which is found in a variety of proteins, may serve a common function as a ubiquitin E3 ligase (for reviews see [38–40]). However, conclusive evidence to demonstrate that PML itself functions as a ubiquitin E3 ligase remains elusive.

Despite recent identification of a number of SUMO-deconjugating enzymes [41–46], there is little information on how these enzymes are regulated. There is also little understanding of how SUMO1 conjugates are processed in the cell. A number of possibilities exist in this regard. SUMO1 could be removed and target proteins then degraded by the standard proteasome pathway, along with other unmodified proteins. Alternatively, as part of their degradation pathway, SUMO1-conjugated proteins could be ubiquitinated on different lysines, becoming co-modified by SUMO1 and ubiquitin. In a further scenario SUMO1 itself could be ubiquitinated leading to SUMO–ubiquitin chains. Particularly for the latter two scenarios, the mechanism of how these conjugates would be dealt with remains an important question. For example, do SUMO-deconjugation enzymes function at this part of the pathway or is the SUMO1 moiety degraded along with the protein to which it is covalently linked? We observed that following proteasome inhibition virtually all of the SUMO1 within the cell (which is mainly nuclear), accumulates in the form of high-molecular-mass conjugates. Upon the reversal from proteasome inhibition, we observed the regeneration of free SUMO1 in the absence of new protein synthesis. These results suggest that SUMO1 deconjugation is involved in the degradation and disposal of SUMO1 conjugates. However, at present we do not know if proteasome activity alone is sufficient to mediate this effect or if, as we suspect, that specific SUMO1 deconjugation

Figure 8 A speculative model on the relationship between the accumulation of ubiquitinated and SUMO1-modified proteins at the NBs

This proposal summarizes one potential model for how the accumulation of SUMO1 conjugates and the appearance of ubiquitin conjugates at the NB may be regulated. In this proposal SUMO1-modified proteins (a) dynamically traffic through the nuclear body (b) wherein SUMO-specific proteases participate in the removal of SUMO1, in a manner which is possibly co-ordinated with the ubiquitination pathway. SUMO1 may be exchanged for ubiquitin either at the same or different lysines and the proteins targeted for degradation by the proteasome (c). MG132 treatment blocks the proteasome resulting in the stacking up of proteins at and around the NB (d).

enzymes participate in this process. Our initial analyses of one of these SUMO-specific proteases, SENP1 (SUMO1/sentrin-specific peptidase 1), indicates that a distinct population of SENP1 localizes to the periphery of PML domains (results not shown), suggesting where it may play a role in this process.

Consistent with this proposal it was recently suggested that SUMO1 modification of PML may actually be a prerequisite for its degradation and necessary for recruitment of the 11 S proteasome subunit to the NB [26]. In that study, increased SUMO1 modification of PML was observed to precede its degradation (although ubiquitinated PML was not itself detected).

A speculative proposal on the relationship between the SUMO1 and ubiquitination pathways is summarized in Figure 8. In this model, the bulk of the SUMO1-modified proteins traffic through the nuclear body (Figure 8a) and the SUMO1 molecule may be removed by a deconjugation enzyme, in a process that can be co-ordinated with the ubiquitination pathway. Whether PML (Figure 8b) participates in this pathway directly, as a ubiquitin E3 ligase, or just serves as a co-ordinating factor remains to be determined. Ubiquitinated proteins then leave the NB to be degraded by the proteasome (Figure 8c). The sorting and fate of cargo at this domain will itself be influenced by the components that are present at the NB at any one time, in a process similar to the dynamic flux that has been proposed for the nucleolus [47]. Blockage of the pathway by MG132 treatment disrupts this flux and results in stacking up of ubiquitinated and SUMO-modified proteins at and around the PML body (Figure 8d). Although we present our favoured model, it remains speculative as we cannot formally exclude other explanations or interpretations of the data, such as those stated previously. It is important to stress that the present study examines the bulk pool of SUMO1-modified species, not the bulk of any individual protein, and the model is based on localization of the bulk SUMO1 pool. It remains the case that for any individual protein, in most studies the majority appears to be in the unmodified form. This may make it difficult to confirm that for any given target protein, it is the SUMO1-conjugated (or ubiquitinated) form which accumulates around the NB during

proteasome inhibition. We do not know, for example, whether SUMO1-modified proteins are co-modified by ubiquitin (both in terms of the general SUMO1 pool and in terms of individual SUMO1-modified proteins). One would need a route to discriminate between the different forms, i.e. unmodified, SUMO1 modified, ubiquitin modified or both. Moreover if these modification events are linked through a regulated feedback mechanism coupled to proteasome activity, then the ubiquitination of SUMO1 modified proteins may be transient and difficult to detect. However certain proposals may be examined. For example, it should be possible to further test the proposal that SUMO1 must be removed prior to processing, using direct *in vitro* assays, comparing the ubiquitination and degradation of individual purified SUMO-conjugated proteins versus unconjugated target species. It may also be possible to develop localization assays to reveal SUMO1 conjugation on specific proteins in live cells, using a fluorescence complementation method, similar to that recently developed [48]. In this procedure the ubiquitin/ubiquitin-like molecule is fused to a non-functional GFP (green fluorescent protein) variant, and the target protein to an interacting but also non-functional GFP variant. When the target protein is covalently modified by the ubiquitin/ubiquitin-like molecule, the non-functional GFP variants are brought into proximity and complement each other, the resulting fluorescent activity specifically reveals the localization of the conjugated species in live cells, such as has been demonstrated for Jun [48]. Further studies on the biochemistry and cell biology of SUMO1 modification in the cell lines generated here should help to elucidate further aspects of the inter-relationship of the PML NBs, SUMO1 modification and deconjugation pathways and proteasome activity.

We are grateful to Paul Freemont (Division of Molecular Biosciences, Imperial College, London, U.K.) for originally supplying the myc-tagged SUMO1 clone (pMLV-myc-PIC1) and for supplying the anti-PIC1 antibody. We thank Dr Roel van Driel (Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands) for the gift of the 5E10 PML monoclonal antibody. This work is supported by Marie Curie Cancer Care.

REFERENCES

- Gill, G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev.* **18**, 2046–2059
- Weissman, A. M. (2001) Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell Biol.* **2**, 169–178
- Okura, T., Gong, L., Kamitani, T., Wada, T., Okura, I., Wei, C. F., Chang, H. M. and Yeh, E. T. (1996) Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin. *J. Immunol.* **157**, 4277–4281
- Boddy, M. N., Howe, K., Etkin, L. D., Solomon, E. and Freemont, P. S. (1996) PIC 1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. *Oncogene* **13**, 971–982
- Matunis, M. J., Coutavas, E. and Blobel, G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* **135**, 1457–1470
- Mahajan, R., Delphin, C., Guan, T., Gerace, L. and Melchior, F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell (Cambridge, Mass.)* **88**, 97–107
- Verger, A., Perdomo, J. and Crossley, M. (2003) Modification with SUMO. A role in transcriptional regulation. *EMBO Rep.* **4**, 137–142
- Johnson, E. S. (2004) Protein modification by SUMO. *Annu. Rev. Biochem.* **73**, 355–382
- Wilkinson, C. R. (2004) Ubiquitin-like proteins: meet the family. *Semin. Cell Dev. Biol.* **15**, 199–200
- Desterro, J. M., Rodriguez, M. S. and Hay, R. T. (1998) SUMO1 modification of I κ B α inhibits NF- κ B activation. *Mol. Cell* **2**, 233–239
- Hoegge, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G. and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature (London)* **419**, 135–141
- Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H. and Weissman, A. M. (2000) MDM2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J. Biol. Chem.* **275**, 8945–8951

- 13 Buschmann, T., Fuchs, S. Y., Lee, C. G., Pan, Z. Q. and Ronai, Z. (2000) SUMO1 modification of MDM2 prevents its self-ubiquitination and increases MDM2 ability to ubiquitinate p53. *Cell* (Cambridge, Mass.) **101**, 753–762
- 14 Maul, G. G. and Everett, R. D. (1994) The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. *J. Gen. Virol.* **75**, 1223–1233
- 15 Matera, A. G. (1999) Nuclear bodies: multifaceted subdomains of the interchromatin space. *Trends Cell Biol.* **9**, 302–309
- 16 Doucas, V. (2000) The promyelocytic (PML) nuclear compartment and transcription control. *Biochem. Pharmacol.* **60**, 1197–1201
- 17 Ruggero, D., Wang, Z. G. and Pandolfi, P. P. (2000) The puzzling multiple lives of PML and its role in the genesis of cancer. *BioEssays* **22**, 827–835
- 18 Zhong, S., Hu, P., Ye, T. Z., Stan, R., Ellis, N. A. and Pandolfi, P. P. (1999) A role for PML and the nuclear body in genomic stability. *Oncogene* **18**, 7941–7947
- 19 Müller, S., Matunis, M. J. and Dejean, A. (1998) Conjugation with the ubiquitin-related modifier SUMO1 regulates the partitioning of PML within the nucleus. *EMBO J.* **17**, 61–70
- 20 Kamitani, T., Nguyen, H. P., Kito, K., Fukuda-Kamitani, T. and Yeh, E. T. (1998) Covalent modification of PML by the sentrin family of ubiquitin-like proteins. *J. Biol. Chem.* **273**, 3117–3120
- 21 Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, III, J. F. and Maul, G. G. (1999) PML is critical for ND10 formation and recruits the PML-interacting protein Daxx to this nuclear structure when modified by SUMO1. *J. Cell Biol.* **147**, 221–234
- 22 Anton, L. C., Schubert, U., Bacik, I., Princiotto, M. F., Wearsch, P. A., Gibbs, J., Day, P. M., Realini, C., Rechsteiner, M. C., Binnik, J. R. et al. (1999) Intracellular localization of proteasomal degradation of a viral antigen. *J. Cell Biol.* **146**, 113–124
- 23 Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M. and Parkinson, J. (1998) The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J. Virol.* **72**, 6581–6591
- 24 Fabunmi, R. P., Wigley, W. C., Thomas, P. J. and DeMartino, G. N. (2001) Interferon gamma regulates accumulation of the proteasome activator PA28 and immunoproteasomes at nuclear PML bodies. *J. Cell Sci.* **114**, 29–36
- 25 Mattsson, K., Pokrovskaja, K., Kiss, C., Klein, G. and Szekely, L. (2001) Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1012–1017
- 26 Lallemand-Breitenbach, V., Zhu, J., Puvion, F., Koken, M., Honore, N., Doubeikovsky, A., Duprez, E., Pandolfi, P. P., Puvion, E., Freemont, P. et al. (2001) Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As₂O₃-induced PML or PML/retinoic acid receptor α degradation. *J. Exp. Med.* **193**, 1361–1371
- 27 Yao, T. and Cohen, R. E. (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* (London) **419**, 403–407
- 28 Bailey, D. and O'Hare, P. (2002) Herpes simplex virus 1 ICP0 co-localizes with a SUMO-specific protease. *J. Gen. Virol.* **83**, 2951–2964
- 29 Batchelor, A. H. and O'Hare, P. (1992) Localization of *cis*-acting sequence requirements in the promoter of the latency-associated transcript of herpes simplex virus type 1 required for cell-type-specific activity. *J. Virol.* **66**, 3573–3582
- 29a Stuurman, N., de Graaf, A., Floore, A., Josso, A., Humbel, B., de Jong, L. and van Driel, R. (1992) A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. *J. Cell Sci.* **101**, 773–784
- 30 Kamitani, T., Kito, K., Nguyen, H. P., Wada, H., Fukuda-Kamitani, T. and Yeh, E. T. (1998) Identification of three major sentrinization sites in PML. *J. Biol. Chem.* **273**, 26675–26682
- 31 Xu, Y., Ahn, J. H., Cheng, M., apRhyas, C. M., Chiou, C. J., Zong, J., Matunis, M. J. and Hayward, G. S. (2001) Proteasome-independent disruption of PML oncogenic domains (pods), but not covalent modification by SUMO1, is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. *J. Virol.* **75**, 10683–10695
- 32 Fujimuro, M., Sawada, H. and Yokosawa, H. (1994) Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett.* **349**, 173–180
- 33 Bloch, D. B., Chiche, J. D., Orth, D., de la Monte, S. M., Rosenzweig, A. and Bloch, K. D. (1999) Structural and functional heterogeneity of nuclear bodies. *Mol. Cell Biol.* **19**, 4423–4430
- 34 Everett, R. D., Lomonte, P., Sternsdorf, T., van Driel, R. and Orr, A. (1999) Cell cycle regulation of PML modification and ND10 composition. *J. Cell Sci.* **112**, 4581–4588
- 35 Bernardi, R., Scaglioni, P. P., Bergmann, S., Horn, H. F., Vousden, K. H. and Pandolfi, P. P. (2004) PML regulates p53 stability by sequestering Mdm2 to the nucleolus. *Nat. Cell Biol.* **136**, 665–672
- 36 Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M. and Parkinson, J. (1997) A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J.* **16**, 1519–1530
- 37 Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A. Y., Qin, J. and Gu, W. (2002) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* (London) **416**, 648–653
- 38 Joazeiro, C. A. and Weissman, A. M. (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* (Cambridge, Mass.) **102**, 549–552
- 39 Tyers, M. and Jorgensen, P. (2000) Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr. Opin. Genet. Dev.* **10**, 54–64
- 40 Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000) Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* (Cambridge, Mass.) **102**, 533–539
- 41 Li, S. J. and Hochstrasser, M. (1999) A new protease required for cell-cycle progression in yeast. *Nature* (London) **398**, 246–251
- 42 Li, S. J. and Hochstrasser, M. (2000) The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. *Mol. Cell Biol.* **20**, 2367–2377
- 43 Schwenhorst, I., Johnson, E. S. and Dohmen, R. J. (2000) SUMO conjugation and deconjugation. *Mol. Gen. Genet.* **263**, 771–786
- 44 Best, J. L., Ganiatsas, S., Agarwal, S., Changou, A., Salomoni, P., Shiriha, O., Meluh, P. B., Pandolfi, P. P. and Zon, L. I. (2002) SUMO1 protease-1 regulates gene transcription through PML. *Mol. Cell* **10**, 843–855
- 45 Gong, L., Millas, S., Maul, G. G. and Yeh, E. T. (2000) Differential regulation of sentrinized proteins by a novel sentrin-specific protease. *J. Biol. Chem.* **275**, 3355–3359
- 46 Kim, K. I., Baek, S. H., Jeon, Y. J., Nishimori, S., Suzuki, T., Uchida, S., Shimbara, N., Saitoh, H., Tanaka, K. and Chung, C. H. (2000) A new SUMO1-specific protease, SUSP1, that is highly expressed in reproductive organs. *J. Biol. Chem.* **275**, 14102–14106
- 47 Andersen, J. S., Lam, Y. W., Leung, A. K., Ong, S. E., Lyon, C. E., Lamond, A. I. and Mann, M. (2005) Nucleolar proteome dynamics. *Nature* (London) **433**, 77–83
- 48 Fang, D. and Kerppola, T. K. (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14782–14787

Received 31 May 2005/21 July 2005; accepted 24 August 2005

Published as BJ Immediate Publication 24 August 2005, doi:10.1042/BJ20050873