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CK2 mediates phosphorylation and ubiquitin-mediated degradation of the PML tumor suppressor

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Abstract The PML tumor suppressor controls growth suppression, induction of apoptosis, and cellular senescence. PML loss occurs frequently in hematopoietic and solid tumors. PML loss often correlates with tumor progression. Casein kinase 2 (CK2) is a stress-activated serine/threonine protein kinase that is oncogenic and frequently overexpressed in human tumor of multiple histological origins. In addition, CK2 overexpression due to gene amplification has been reported to be an adverse prognostic factor in non-small cell lung cancer. At the 5th International Conference on Protein Kinase CK2 in Padova, Italy, we reviewed our recent findings that PML undergoes ubiquitin/proteasome-mediated degradation in immortalized and tumor derived cell lines. PML degradation depends on direct CK2 phosphorylation of PML Ser517. PML mutants that are resistant to CK2 phosphorylation display increased tumor suppressive functions in assays measuring apoptosis, replicative senescence, and in xenograft models. More significantly, CK2 pharmacological inhibition enhances PML tumor suppressive property. These data identify a key post-translational mechanism that controls PML protein levels in cancer cells and suggest that CK2 inhibitors may be beneficial anti-cancer drugs.

Keywords CK2 · PML · Protein polyubiquitination · Lung cancer pathogenesis

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Introduction

The promyelocytic leukemia tumor suppressor (PML), initially identified as a component of the PML-RARα oncoprotein of acute promyeolocytic leukemia (APL), plays a critical role in apoptosis regulation, oncogene induced senescence (OIS) induction, and response to DNA damaging agents. PML is ubiquitously expressed and mainly localizes to distinct nuclear structures identified as PML nuclear bodies (PML-NBs). PML is a member of the RING-B-box-coiled-coil (RBCC) protein family and contains three zinc finger-like domains, a coil-coiled dimerization domain, a nuclear localization signal and a C-terminal degron. The RBCC region mediates protein/protein interactions and is essential for PML localization to the PML-NBs. PML is post-transcriptionally modified by sumoylation and phosphorylation. Sumoylation is essential for proper assembly of the PML-NBs and specific phosphorylation events mediate DNA damage induced apoptosis and/or degradation [1, 2].

Several lines of evidence indicate that PML is a tumor suppressor. PML-RARα plays a causal role in APL pathogenesis by disrupting PML-NBs and inhibiting PML function [3, 4]. Reduction of PML dose results in acceleration of leukemia onset in a transgenic APL mouse model, and *Pml* knockout mice are highly susceptible to tumor development when challenged with physical and

chemical carcinogens. *Pml* loss results in striking defects in OIS and apoptosis induction due to loss of sensitivity to p53 dependent and independent stimuli, such as ceramide, gamma radiation, and type I and II interferons. In addition, *Pml* inactivation protects fibroblasts from UV-induced apoptosis in a p53 independent manner [5–8]. Furthermore, *Pml* loss markedly accelerates tumor onset, incidence, and progression in an onc-K-Ras induced NSCLC mouse model and in *Pten*-heterozygous mutant mice [1, 9].

At the molecular level, PML interacts with the tumor suppressor p53 and transcriptional co-activator CBP/p300 (cAMP response element-binding protein) as a result of a specific protein sequence present at its C-terminus [10]. Other PML interacting proteins relevant in tumor suppression are the tumor necrosis factor receptor interacting protein DAXX, the phosphatase PP2a, mTOR, and Smad4 [9, 11, 12].

CK2 is a nuclear matrix associated, highly conserved, and ubiquitous serine/threonine kinase that consists of two catalytic ($\alpha\alpha$,α' α' or $\alpha\alpha'$) and two β regulatory subunits [13]. Traditionally, CK2 has been considered a constitutively active and nonregulated protein kinase. However, there is a growing consensus that CK2 plays a critical role in regulating cell survival [14]. Several studies indicate that CK2 activity is required for cell viability in baker's yeast, nematode, and mouse [14–17]. CK2 is oncogenic in transgenic mice and is overexpressed in several human cancers. CK2 overexpression predicts poor survival in NSCLC [14, 18–20]. These results strongly suggest that CK2 plays a causal role in tumorigenesis. Consistent with this suggestion, CK2 phosphorylates and positively modulates the activity of several proteins that play a critical role in cell survival. For example, CK2 phosphorylation leads to the ubiquitin/proteasome-mediated degradation of IκBα, an inhibitor of the prosurvival transcription factor NF-kB [21]. Other examples are represented by the oncogene AKT whose activity is positively regulated by CK2 phosphorylation, β-catenin whose phoshorylation promotes its translocation to the nucleus, and Bid whose activation is inhibited by CK2 phosphorylation [22–24].

Others and we have reported that PML protein is completely or partially lost in a large fraction of human cancers and that this loss correlates with tumor progression. In these cases, sequence analysis of the PML gene and promoter methylation studies did not detect any inactivating mutations or aberrant methylation. However, we consistently detected PML mRNA transcripts by in situ hybridization in these tumor samples. These data suggest that PML loss is mediated at the post-transcriptional level and led to the hypothesis that it is aberrantly degraded in human cancer [25–27]. We hypothesized that the mechanisms underlying PML protein loss in human cancer play an important role in tumorigenesis and sought their identification.

Results

PML undergoes ubiquitin proteasome-mediated degradation

At the 5th International Conference on Protein Kinase CK2 (September 14–16, 2007 in Padova, Italy), we discussed our recent studies regarding the aberrant PML protein polyubiquitination that occurs in tumor cells. We described that PML undergoes ubiquitin/proteasome-mediated degradation upon a direct phosphorylation event mediated by CK2.

PML undergoes protein polyubiquitination and proteasome dependent degradation in immortalized and tumor derived cell lines, such as NIH3T3, HEK 293, and ColoDM220 but not in primary mouse embryonic fibroblasts. To map the protein sequence necessary and sufficient to direct PML ubiquitinylation, we generated a large series of PML deletion mutants that we screened upon transfection with HA-ubiquitin in HEK293 cells for the ability to be conjugated to polyubiquitin chains. This analysis led to the discovery that a discrete C-terminal PML protein sequence (PML 498–525) is necessary to allow PML protein polyubiquitination and degradation. Moreover, PML 498–524 is sufficient to direct polyubiquitinylation of a heterologous protein, such as GFP. A Summary of these results is provided in Fig. 1. We concluded that this sequence is the PML degron.

CK2 phosphorylates the PML degron directly

We identified multiple phosphorylation consensus sites for CK2 in the PML degron (Fig. 1). PML S514 is a low probability CK2 consensus site in unmodified PML that becomes a high probability CK2 consensus site when S517 is phosphorylated [28, 29]. Indeed, we found that 6His-tagged full-length Casein kinase 2α produced in baculovirus readily phosphorylates bacterially expressed PML by an immunocomplex kinase assay. Site directed mutagenesis experiments followed by in vitro kinase assays performed in the presence of specific CK2 inhibitors, such as TBB and DMAT indicated that PML S517 is the primary CK2 phosphorylation site and suggested that phosphorylation of this serine primes phosphorylation of S514. These conclusions were confirmed by MALDI-re-TOF mass spectrometry experiments on recombinant PML proteins phosphorylated in vitro [1].

These experiments led to the conclusion that PML S517 is the major CK2 acceptor site.

p38 MAPK activation is required for PML degradation

Since CK2 activation depends on p38 MAPK during cellular stress, we investigated whether p38 MAPK activation leads

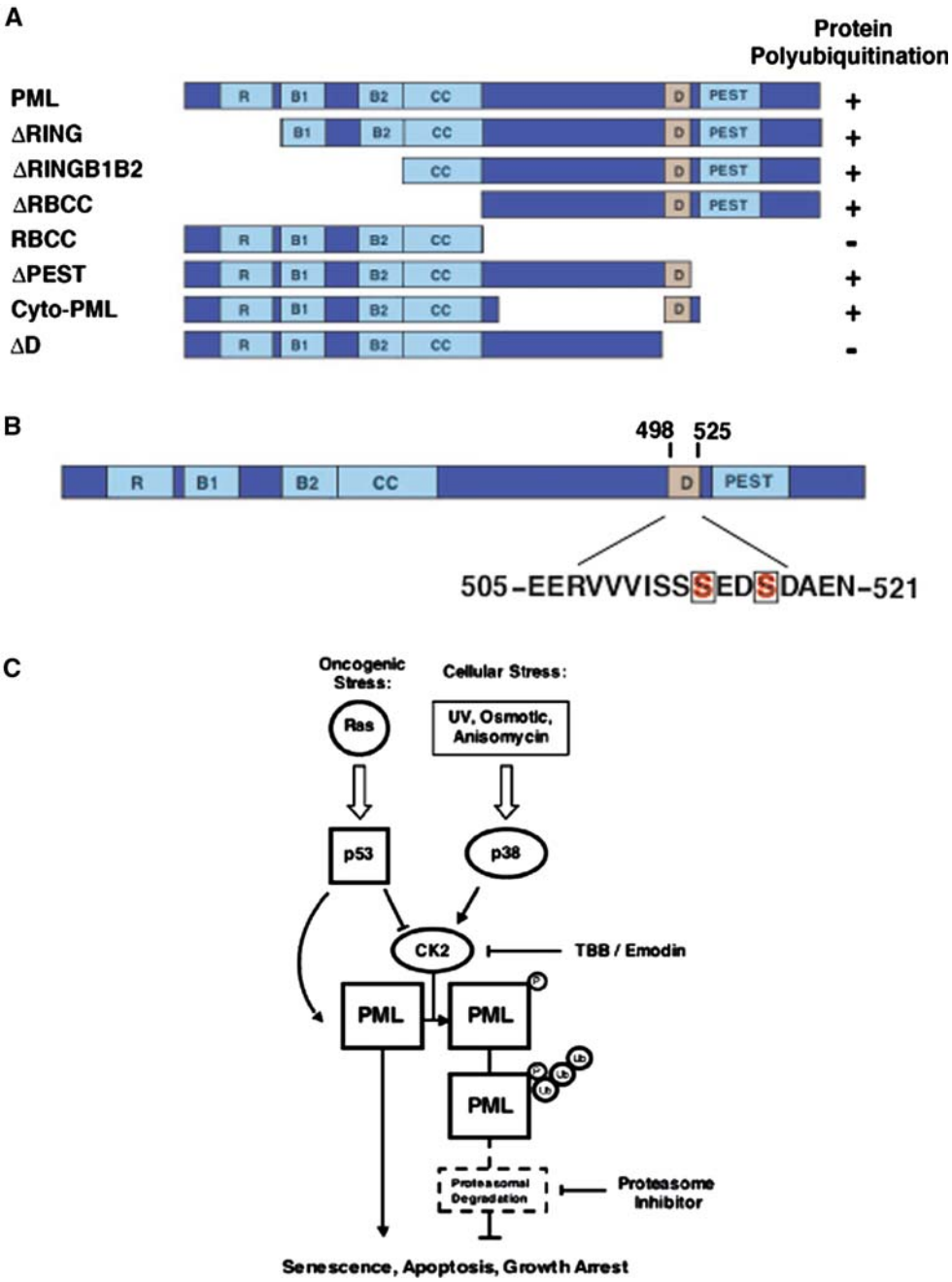

Fig. 1 PML is ubiquitinated and degraded upon CK2 phosphorylation. (**a**) Schematic representation of informative PML constructs used in the studies presented at the 5th International Conference on Protein Kinase CK2. PML modular organization is represented along with its major domains. Boxes represent PML domains. R: Ring finger; B1 and B2: B-Boxes: CC: Coiled-Coil; D: Degron; PEST: PEST domain. The column on the right indicates whether mutant PML proteins undergo polyubiquitinylation. (**b**) Schematic representation of PML serines 514 and 517 (indicated by boxes), the CK2 phosphoacceptor sites we identified in this study. (**c**) Molecular mechanisms controlling PML protein polyubiquitination. In primary cells, oncogenic stress induces the p53 tumor suppressor, which in turn induces PML and downregulates CK2 activity, leading to a tumor suppressive response. In this model, CK2 is part of a prosurvival network that allows cell survival in the context of cellular stress. CK2 deregulation (for example due to gene amplification in NSCLC) leads to tumorigenesis due to degradation of the PML tumor suppressor. In this situation, therapy with CK2 or proteasome inhibitors will abrogate aberrant PML protein degradation, leading to restoration of PML tumor suppressive properties

to PML polyubiquitinylation and degradation [21, 30]. We found that several treatments that activate p38 MAPK, such as osmotic shock, anisomycin, and UV radiation, lead to PML protein polyubiquitination and degradation in NIH 3T3 cells. Next, we found that in the context of osmotic shock, inhibition of p38 MAPK with p38AF, a dominant negative mutant, or the specific inhibitor SB202190 efficiently blocks PML degradation in NIH 3T3 cells. Thus, we concluded that PML degradation induced by osmotic shock is dependent on p38 MAPK activity.

CK2 is required for PML degradation

We found that TBB and TBCA (two specific CK2 inhibitors) abrogate osmotic shock induced PML degradation and that two specific siRNA oligonucleotides caused a 75% reduction in CK2α protein in NIH 3T3 cells. In this setting, endogenous PML protein was more than twice the level observed in cells treated with scrambled siRNA. These experiments confirm that CK2 kinase is required for PML degradation.

Furthermore, we found that abrogation of PML S517 resulted in complete resistance to sorbitol-induced degradation, These experiments further validate that PML phosphorylation by CK2 of PML S517 is essential for its degradation [1].

Mutations at S517 affect PML stability and tumor suppressive function in vitro and in vivo

PML is a tumor suppressor protein capable of inducing growth arrest and apoptosis [2]. Therefore, we hypothesized that PML S517A, the mutant refractory to CK2-mediated phosphorylation and ubiquitin-mediated degradation, acts as a super-tumor suppressor due to its resistance to CK2-mediated degradation. In line with this hypothesis, we found that the PML S517A mutant induces replicative senescence in WI38 human primary fibroblasts and UV induced apoptosis in MEFs unlike wild-type PML.

We then examined the tumor suppressive role of PML S517A as compared to wild-type PML in vivo. To this end, we utilized Colo320DM cells, where PML is degraded in a CK2 dependent manner, that stably expressing either wild-type or PML S517A proteins. Transduced Colo320DM cells were injected subcutaneously into athymic nude mice and xenograft growth quantified. These experiments indicated that growth of Colo320DM cells expressing PML S517A is reduced by more than 50% when compared to cells expressing wild-type PML [1].

These data demonstrate that PML S517A behaves as a super active PML mutant due to defective CK2 mediated degradation.

CK2-dependent degradation of PML in tumor derived cell lines and in human NSCLC

PML is often partially or completely lost in non-small cell lung cancer (NSCLC) [1, 25, 27], while CK2 is overexpressed and amplified in NSCLC [18]. Therefore, we tested for an inverse correlation between the two in a panel of NSCLC cell lines and primary human NSCLC specimens. We found that PML protein was barely detectable in A549, H1299, and H322 cells. On the contrary, PML protein was easily detected in H2030, H157, H1975, H1650, and H358 cells. We performed a CK2 kinase assay on H1299 and H322 and on H1650 and H358 (representative of cells with high and low PML protein levels, respectively) cells. CK2 kinase activity was strikingly elevated in H322 and H1299 as compared to H1650 and H358 cell lines.

We also found an inverse correlation between PML protein levels and CK2 kinase activity in primary NSCC specimens. We evaluated CK2 kinase activity and PML protein levels in 18 primary NSCLC specimens and their unaffected counterpart tissue that were snap frozen at the time of their surgical resection. PML protein was reduced by at least 50% in 10 out of the 18 tumors analyzed, as compared to the unaffected tissue. CK2 kinase activity was increased by at least 50% in nine of these tumors. Therefore, we found a strong association between elevated CK2 kinase activity and decreased PML protein level ($P = 0.002$). These observations strongly suggest that elevated CK2 kinase activity leads to PML degradation in primary human NSCLC.

Pharmacologic inhibition of CK2 leads to a significant anti-tumor effect in vivo

We tested whether CK2 pharmacologic inhibition led to significant anti-tumor effects. For these experiments, we used emodin, a specific CK2 inhibitor, to treat nude mice bearing Colo320DM xenografts [31]. As expected, emodin upregulated PML, but not PML S517A in cultured Colo320DM cells. Treatment with emodin in vivo reduced the tumor burden by more than 50%. These results suggest that pharmacologic inhibitors may have anti-tumor properties [1].

Discussion

Traditionally, CK2 has been regarded as a constitutively active serine/threonine protein kinase in search of specific physiological functions [13]. However, several studies have indicated that CK2 plays a critical role in the regulation of cell proliferation and survival [14]. The molecular pathways modulating the pro-survival properties of CK2 are being unraveled and it is becoming apparent that CK2 modulates several pathways that play a key role in regulating apoptosis and cell growth (refer to other sections of this proceedings book) For example, CK2 is activated by UV in a p38 MAPK dependent manner, leading to phosphorylation and degradation of the NF-κB inhibitor IκBα [21]. Furthermore, upon UV irradiation CK2 complexes and phosphorylates p53 at S389. MEF cells and mice carrying the p53 S389A mutant in the p53 locus have defects in the induction of p53 target genes, apoptosis, and increased skin tumorigenesis upon UV [32–34]. Conversely, wild-type p53 inhibits CK2 protein kinase [35]. These observations support the notion that p53 and CK2 functions are interconnected in a tightly regulated network. An explanation of these apparently conflicting results is that the activation of NF-κB by UV radiation may promote p53-mediated cell cycle arrest. In this context, CK2 would exert a prosurvival activity that allows repair of genotoxic insults.

PML has been the subject of intense investigation due to its multiple tumor suppressive functions and its ability to

regulate key tumor suppressive pathways. Our findings define the first pathway that negatively regulates PML levels in both oncogenic and physiological conditions and establish that pharmacologic CK2 inhibition restores PML function counteracting tumorigenicity in vivo.

In mammalian cells, p38 MAPK is strongly activated in response to stress stimuli ranging from osmotic shock, UV, ionizing radiation, and inflammatory cytokines. Activation of p38 MAPK in turn leads to upregulation of CK2 kinase activity [21, 36]. This pathway plays an essential role in regulating inflammation, cell differentiation, cell growth, and apoptosis. The p38 MAPK pathway is generally regarded as pro-apoptotic. However, there is evidence that p38 MAPK activation can also protect from apoptosis during conditions of cellular stress and may contribute to human tumorigenesis [21, 37–39].

Our work defines a functional network between p38 MAPK, p53, CK2, and PML (Fig. 1). In addition to its well-established role as a p53 co-activator during genotoxic stress, PML regulates UV response by inducing apoptosis or cell cycle arrest in a p53-independent manner [2]. In this context, the phosphorylation of PML by CK2 (and the concomitant phosphorylation of IκBα by CK2) is part of a cellular circuitry that attenuates apoptosis, allowing cells to recover from noxious stimuli.

In conditions of oncogenic stress, such as the ones triggered by oncogenic Ras, PML is activated and exerts its tumor suppressive function in concert with several partners including p53. In this context, p53 may inhibit CK2 to achieve maximal PML activity and tumor suppressive effects. On the contrary, when CK2 kinase activity is upregulated (as often happens in human cancers), PML is polyubiquitinylated and degraded (Fig. 1). This scenario may be particularly relevant for the pathogenesis of NSCLC where increased CK2 kinase activity may occur because of either p38 MAPK activation or CK2α gene amplification, a marker of poor prognosis in this disease [18].

We propose that therapy with specific CK2 inhibitors or proteasome inhibitors, such as bortezomib, which is currently used in the treatment of several human cancers [40, 41], may be particularly effective in tumors that display aberrant CK2 activity and loss of PML protein.

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