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Induction and activation of the p53 pathway: a role for the protein kinase CK2?

David W. Meek · Miranda Cox

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Abstract Protein kinase CK2 has many established in vitro substrates, but it is only within the past few years that we have begun to ascertain which of these are its real physiological targets, how their phosphorylation may contribute towards regulating normal cell physiology, and how phosphorylation of these proteins might influence the development of diseases such as cancer. One of the well-characterised in vitro substrates for CK2 is the tumour suppressor protein, p53. However, the physiological nature of this interaction has never been fully established. In the present article, we summarise a recent study from our laboratory showing that phosphorylation of p53 at Ser392, the sole site modified by CK2 in vitro, is regulated by a novel mechanism where the stoichiometry of phosphorylation is governed by the rate of turnover of the p53 protein. Such a model is entirely consistent with phosphorylation by a constitutively active protein kinase such as CK2. In contrast to this, while there is overwhelming evidence that CK2 phosphorylates p53 in vitro and is the only detectable Ser392 protein kinase in cell extracts, our data raise uncertainty as to whether this interaction truly reflects events underpinning Ser392 phosphorylation in vivo. We consider the possible role of CK2 in regulating the p53 response in a wider context and suggest key issues that should be addressed experimentally to provide a more cohesive picture of the relationship between this important protein kinase and a pivotal anti-cancer surveillance system in cells.

D. W. Meek (✉) · M. Cox
Biomedical Research Institute, Ninewells Hospital, University of Dundee, Dundee DD1 9SY, UK
e-mail: d.w.meek@dundee.ac.uk

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Introduction

The p53 pathway

The p53 tumour suppressor is a short-lived transcription factor, which plays a critical role in eliminating tumour cells by coordinating changes in gene expression, leading to cell cycle arrest, senescence or apoptosis [1–3]. In addition to its well-established role in cancer, it is also becoming increasingly clear that basal p53 levels can influence a variety of important physiological events including metabolism, ageing and development, and that the involvement of p53 in some of these events may potentially contribute towards tumour suppression (for example, suppression of the Warburg effect) [2, 3].

p53 is induced in response to various tumour-related stresses, mainly through uncoupling from its key negative regulators, MDM2 and MDM4, which results in the accumulation of stable active p53. Importantly, MDM2 is one of several ubiquitin ligases that can mediate the ubiquitylation and proteasome-dependent degradation of p53, both homeostatically in normal unstressed cells and as a means of restoring p53 levels after reversible activation of the p53 pathway. While other E3 ligases can contribute to regulating p53 levels, it is overwhelmingly clear that MDM2 plays a pivotal role in this process.

Protein kinase CK2

The serine/threonine protein kinase CK2 is a highly conserved tetrameric enzyme composed of two catalytic (α

and/or α') subunits tethered together by two regulatory (β) subunits [4, 5]. CK2 plays a critical role in many cellular processes and regulates several important growth/survival pathways including the PI3K/Akt, WNT, NFκB and DNA damage response pathways [5]. CK2 has been described as a 'non-oncogene': a term that signifies that it does not undergo activation by mutation as many other growth/survival-associated protein kinases do, yet its levels and activity are increased in a range of different types of cancer (e.g. through gene amplification) and, at least in some cases, are associated with a reduction in patient survival [5]. Elevated CK2 has pro-survival and anti-apoptotic consequences and current perception is that cancer cells can become 'addicted to' or dependent upon elevated CK2 for their survival. Consistent with this idea, an orally bioavailable small molecule inhibitor of CK2 gives rise to anti-proliferative and anti-angiogenic responses in murine tumours and has found its way into clinical trials [6].

CK2 is constitutively active, at least when measured in cell extracts, and a wide range of substrates have been identified for this kinase in vitro [7]. It is still uncertain whether or how CK2 is controlled or its activity regulated, but it is not activated or repressed as part of a classical protein kinase cascade such as the ERK pathway [5]. Phosphorylation of target proteins by CK2 could be regulated through the involvement of other kinases such as p38 MAPK (e.g. see [8]) although the mechanism by which this might occur is unclear. Alternatively, CK2-mediated phosphorylation may be regulated at the de-phosphorylation step or by recruitment to specific complexes. For example, the regulation of CK2 activity and specificity towards the Ser392 site in p53 (discussed below) was reported to occur through association with FACT (facilitates chromatin transcription) complex [9]. Moreover, it has been proposed that FACT–CK2 complex formation is induced in response to ultraviolet radiation (UVC) with the outcome of altering the conformation of CK2 and causing increased specificity towards p53 (Keller and Lu 2002). However, this model has yet to be investigated in vivo.

Regulation of p53 by multisite post-translational modification

p53 is reversibly modified through several mechanisms including phosphorylation, acetylation, glycosylation, methylation, neddylation, sumoylation, and ubiquitylation. These events occur mainly within the N- and C-termini, and are thought to perform a variety of important functions from regulating p53 stability to activating its tumour-suppressing transcriptional activities. Many of these modifications occur in response to stress stimuli such as UV irradiation and DNA strand breaks. Their functions and regulations have been reviewed extensively elsewhere [10–13].

Phosphorylation of p53 by protein kinase CK2

The phosphorylation of p53 by protein kinase CK2 was first demonstrated more than 20 years ago by two independent groups [14, 15]. The penultimate residue in the p53 protein, Ser392 in human p53 (Ser389 in murine p53), was identified as the phospho-acceptor residue, and both the association of p53 with CK2 in immunoprecipitation experiments, plus the finding that the sole Ser392 kinase activity detectable in cell extracts co-purifies with CK2, provided strong, but not conclusive evidence that their association was likely to be physiological. Interestingly, Ser392 is not embedded within a classical CK2 consensus site but is flanked on its C-terminal site by a single aspartate residue (which, being at the C-terminus, provides two negative charges) as opposed to the cluster of tandem acidic amino acids normally associated with CK2 phosphorylation sites. Nevertheless, from an evolutionary perspective, this is a highly conserved residue in p53, strongly underpinning its potential importance for the function or regulation of the protein. In addition to a proposed catalytic role for CK2 in phosphorylating p53, the regulatory β-subunit of CK2 can interact with p53; in contrast to phosphorylation by CK2 (see below), this protein–protein interaction leads to reduced DNA binding and transactivation by p53 [16, 17]. Reciprocally, the interaction of p53 with the regulatory (non-catalytic) β-subunit of CK2 may lead to inhibition of CK2 protein kinase activity [18]. The relationship between these two proteins may, therefore, be more complex than simply an enzyme/substrate interaction.

The importance of Ser392 in regulating p53 function has been highlighted in both in vitro and in vivo studies. At the biochemical level, phosphorylation of Ser392 activates the site-specific DNA-binding function [19] and tetramerisation of p53 [20]. It can also play a positive role in promoting p53-mediated suppression of colony formation by cultured cells [21]. From a biological perspective, insight has been gained from the generation of mice that express p53 proteins in which Ser389 is substituted by alanine. These animals show selective susceptibility to UV-induced skin tumours [22] and chemically induced bladder tumours [23]. Moreover, cells from these mice show alterations in UV-induced p53 levels, site-specific DNA binding, and notable differences in the expression of hundreds of p53-targeted genes [22, 24]. Collectively, these data support the idea that modification of this site has important functional consequences for p53. It is also interesting that the phosphorylation of Ser392 has an impact on mutant p53 [25]. Mutation of the *TP53* gene is a common event during tumour development with the outcome that many mutant

p53 proteins become stable and acquire dominant-negative or even 'gain-of function' status, both of which may contribute mechanistically to the development of the tumours [26]. Ser392 phosphorylation can sensitise certain p53 mutants to degradation, indicating that it may have an important impact on the oncogenic properties of these proteins [25].

In addition to its phosphorylation by CK2, Ser392 is reported to be a target of several other protein kinases in vitro including: p38MAPK [27, 28], the interferon-activated dsRNA-dependent protein kinase PKR [29], CDK9 [30, 31] and an as yet unidentified protein kinase that is resistant to the CK2-selective inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) [32]. In all of these cases, however, while the biochemical and cell-based data are encouraging, it is far from clear whether modification of this site is mediated by one or more of these different protein kinases, under any given set of circumstances.

Phosphorylation of Ser392 in response to specific stimuli

Ser392 phosphorylation was reported to occur preferentially in response to UV radiation [33–35]. However, this may be an over-simplification because evidence for increased phosphorylation in response to other p53-inducing stimuli, such as ionising radiation, is evident in the published data of other groups (e.g. see [35, 36]). One difficulty in assessing whether the phosphorylation of Ser392 varies under any given set of circumstances is that increased p53 levels and stability occur in response to most, if not all, stimuli that induce the p53 pathway. It has therefore been difficult to discern whether observed increases in the levels of Ser392 phosphorylation truly represent increases in the stoichiometry of phosphorylation or merely reflect a proportionate increase in the level of p53 protein.

In order to address this issue, we quantified the level of Ser392 phosphorylation (using two independent Ser392-targeted phospho-specific antibodies) relative to total p53 levels in a variety of different cell lines and primary human cells, and in response to a range of stimuli that induce the p53 pathway. We made several interesting and important observations (recently published in [37]): (1) We noted that Ser392 phosphorylation occurs at a relatively low level in growing cells, under normal unstressed conditions. Such constitutive phosphorylation is certainly consistent with the targeting of p53 by a constitutively active protein kinase such as CK2 and suggests that basal, uninduced p53 is under regulation by this post-translational modification. (2) We noted a significant increase in the stoichiometry of Ser392 phosphorylation, not only in response to increasing doses of UVC, but also after treatment with ionising radiation, the topoismerase II inhibitor etoposide (which induces double strand DNA breaks), the MDM2 inhibitor Nutlin-3, or expression of the ARF protein (a physiological MDM2 inhibitor that is normally induced by unregulated oncogene activity). Nutlin-3, which gave the most potent response, is a particularly interesting case because this inhibitor simply blocks the interaction of the N-terminus of p53 with its binding pocket in the N-terminus of MDM2, leading to impaired p53 degradation. Moreover, unlike the other stimuli used, both ARF and Nutlin-3 do not lead to changes in the phosphorylation status of key N-terminal sites in p53 [38, 39]. The finding that Ser392 phosphorylation can be induced by as much as fivefold simply by uncoupling p53 from its principal negative regulator suggests that (a) regulation of phosphorylation of Ser392 is likely to occur through a mechanism that is common to all of the stimuli used (because all the stimuli lead to uncoupling of p53 and MDM2); and (b) given that one of the key features of MDM2 is that it regulates the stability of p53, p53 stabilisation may have a profound influence on the level of Ser392 phosphorylation that can be achieved.

A model to explain the increase in Ser392 phosphorylation upon p53 induction

To explain our findings, we propose that, under normal physiological conditions, where the turnover of p53 is rapid, phosphorylated p53 molecules are unlikely to accumulate assuming that the rate of phosphorylation is slow (see Fig. 1). However, in response to agents that stabilise p53, the extended half-life would permit the proportion of molecules that are phosphorylated to increase and accumulate for two reasons (i) that the p53 molecules would persist long enough for the slow rate of phosphorylation to make an impact and; (ii) that the rate of phosphorylation is proportional to the substrate concentration. To test this idea we treated cells with the proteasome inhibitor, MG132, to block p53 degradation, then measured the relative change in Ser392 phosphorylation. Under these circumstances, Ser392 phosphorylation was indeed observed to increase, thereby supporting our hypothesis [37]. One approach that could underpin this model significantly would be to inhibit the Ser392 kinase, then subsequently remove the inhibitor and assess whether indeed the rate of phosphorylation is slow. However, this cannot be achieved until the identity of the physiological Ser392 kinase(s) has been established with certainty (see below).

The value of such a general mechanism is that it is immediately brought into play upon p53 induction and is not restricted to any given stimulus or activating pathway. As such we propose that it is thus a common and integral event in activation of the p53 response. The universality of

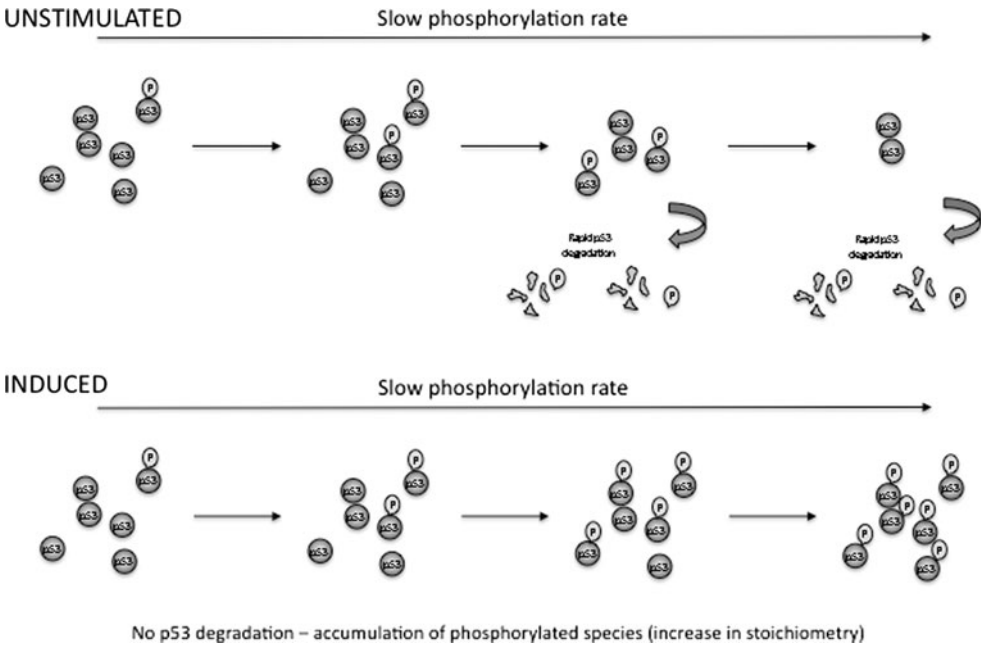

Fig. 1 Model describing the regulation of p53-Ser392 phosphorylation through changes in the turnover of the p53 protein. Details of the model are discussed in the text

this model is underscored by the fact that all stresses/stimuli that induce p53 do so, at least in part, by stabilising p53. We predict, therefore, that increased Ser392 phosphorylation should occur in all the cases. This model also explains why Ser392 phosphorylation is observed after infection or transformation by viruses such as SV40 where the outcome of association with viral proteins such as the large T antigen is the stabilisation of p53 [40].

Does protein kinase CK2 phosphorylate p53 in a physiological context?

Based on our observations that Ser392 phosphorylation can be induced by diverse stimuli in addition to UV, we re-examined the generally accepted idea that modification of this site is mediated by the p38 pathway, potentially through a mechanism in which UV-induced p38 activation leads to the recruitment of CK2 [27, 28, 41]. To address this, we took two complementary approaches. In the first, we phosphorylated p53 in vitro using either recombinant protein kinase CK2 or recombinant p38 [37]. We then analysed the in vitro phosphorylated products by two-dimensional phosphopeptide mapping. This approach showed clearly that p38 cannot phosphorylate Ser392, thereby eliminating this protein kinase as a Ser392-modifying enzyme. In the second approach, we activated the p53 pathway using diverse stimuli in the presence and absence of the widely used p38 inhibitor, SB203580. Under these conditions, we were able to confirm inhibition of p38 in cultured cells, but we did not observe any change in the modification status of Ser392 [37]. These analyses strongly suggest that p38 does not lead to the phosphorylation of Ser392 in cultured cells either directly or indirectly.

As part of these analyses, we also determined whether elimination of CK2 activity, either using the inhibitor DMAT, or by silencing the expression of the CK2α and -α' subunits, affected Ser392 phosphorylation. Surprisingly, neither of these treatments led to any reduction of Ser392 phosphorylation. Therefore, although CK2 appears to be the sole detectable Ser392-targeted protein kinase detectable in cell extracts and upon purification (e.g. see [15]), these new data suggest that it is highly unlikely that it mediates Ser392 phosphorylation in a biological context. These conclusions are in strong agreement with those from another recent study [42]. There is one caveat to this conclusion, however. We had previously published data strongly suggesting that the *dephosphorylation* of Ser392 is an extremely slow process [43]. Assuming this to be the case, it might not be possible to discern any changes in phospho-Ser392 levels in cultured cells simply by inhibiting the enzyme(s) that modifies this site because most of the p53 molecules are already likely to be phosphorylated at this site. This issue will need to be addressed in any future study of this modification event.

A role for CK2 in regulating the p53 pathway?

The data discussed above raise a number of issues that will need to be addressed to fully establish whether p53 is truly a physiological target of CK2. From a more biological perspective, it is worthwhile considering the potential impact of CK2 signalling on the p53 response and how the interaction between these two molecules might fit into this context. For example, it is now becoming clear that, at least in a general sense, protein kinase CK2 is anti-apoptotic and is considered to be a driver of cancer progression [5]. One

possibility is that, as CK2 levels and/or activity increase in a developing cancer cell, increased phosphorylation of Ser392 may be part of the mechanism by which p53 senses oncogenic changes in the cellular environment. Such a model would predict that (a) tumours that show elevated CK2 levels should also show increased p53-Ser392 phosphorylation; and (b) mice lacking the CK2 phosphorylation site (Ser389 in mouse p53) should show increased tumour susceptibility in cases where the tumours have elevated CK2 levels. Both of these predictions should be testable. It has also been shown that Ser392 phosphorylation can sensitise certain p53 mutants to degradation [25]. One might therefore predict that, in tumours expressing such mutant p53 proteins, elevated CK2 would lead to a decrease in the levels of the mutant p53 and consequently alleviation of their tumour-promoting activity. Again, however, this would need to be tested by experimentation.

In addition to p53 itself CK2 is likely to impinge upon other components of the p53 network. For example, besides ourselves others showed that Ser269 in MDM2 is a target for phosphorylation by CK2 in vitro [44]. We also showed that substitution of this residue by alanine led to a decrease in the ability of MDM2 to mediate the turnover of p53 in cultured cells. These data suggested to us that constitutively active CK2 is likely to promote or sustain MDM2 function under normal, unstressed conditions. The finding that treatment of cells with the CK2 inhibitor, DMAT, blocks the appearance of characteristic ubiquitylated forms of p53 [37, Fig. 5A] is consistent with this idea and suggests that CK2 regulates p53 ubiquitylation. In addition, Ser269 lies within a highly acidic region in MDM2 that is modified by GSK3β and protein kinase CK1 at several key residues that promote the turnover of p53 [45–49]; there is a strong possibility, therefore, that regulation of MDM2 by CK2 is not a 'stand alone' event but may synergise with these neighbouring modifications to bring about an integrated response to changes in the cellular environment. However, the stimulation of MDM2 function is at odds with the idea that CK2, if it is a true physiological modifier of p53, phosphorylates a site in p53 that leads to its activation. This is a key issue that will need to be addressed. The outcome of elevated CK2 activity in tumours will also need to be considered. Such increased activity might be expected to augment MDM2 function leading to suppression of p53. This would be particularly relevant given that increased MDM2 expression is a feature of several tumour types and is thought inhibit p53 tumour suppressor function [50]. Future analysis of the relationship between CK2 and the p53 pathway should take these key issues into account.

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