VRK1 interacts with p53 forming a basal complex that is activated by UV-induced DNA damage

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1. Introduction
Cellular reaction to DNA damage is mediated by p53 [1] that triggers specific biological responses based on the physiological situation of individual cells [2,3]. Mainly, the activated p53 protein signals either to protect cells by arresting the cell cycle and to permit DNA repair, or alternatively to induce cell death, thus protecting the organism by elimination of damaged cells [4,5]. In order to initiate these responses cells need to have a basal system, in which proteins participating in damage sensing are organized in a way that will permit their immediate reaction DNA damage detection. This readiness state also requires that cells must always have a basal level of p53 protein that can be immediately stabilized and activated in response to local detection of DNA damage. This immediate p53 stabilization is performed by kinases that phosphorylate p53 in several specific residues, which play different and sequential functional roles [6]. Initial p53 phosphorylation determines the switch between two alternative p53 binding modes, required for either p53 degradation by ubiquitin-ligases, or alternatively p53-dependent transcription. This functional switch is regulated by a unique phosphorylation in p53 in Thr-18 that is mediated by vaccinia-related kinase 1 (VRK1) [7–9]. Structurally, p53-Thr18 forms a hydrogen bond with Asp21 that is necessary for the correct folding of a hydrophobic α-helix and required for the p53 direct interaction with a hydrophobic pocket in mdm2 [10]. p53-Thr18 phosphorylation disrupts this hydrogen bond destabilizing the α-helix and resulting in loss of p53 binding to hdm2/mdm2 [8,11–13]. Furthermore, Thr18 phosphorylation determines a threshold of p53 transcriptional activation [11,14]. Additional p53 phosphorylation mediated by other kinases [6], mainly in residues Ser15 or Ser20, determine the selection and binding specificity of p53 to different transcriptional cofactors [11,12], such as p300 or pCAF [15].

The VRK gene family appeared late in evolution and is composed of three members in mammals [16,17], one member in

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invertebrates, such as Drosophila melanogaster [18] and Caenorhabditis elegans [19], and none in yeast. This VRK gene complexity is similar to that of the p53 gene family [20,21]. Thus, both VRK and TP53 gene families have a parallel evolution, suggesting that their functions might be associated, and thus represent the acquisition of new levels of regulation, which are required as organisms became more complex. The functional connection between VRK1 and p53 was already detected in germ cell proliferation in C. elegans, in which Vrk-1 regulates cell cycle arrest triggered by CEP-1 (p53) [22].

VRK1, the most abundant Ser–Thr kinase in nuclei [23], is a nucleosomal/chromatin kinase that can form complexes and phosphorlyate several transcription factors [24–26], histones [18,27–29] and other chromatin proteins [30,31]. Moreover, VRK1 kinase activity can also be regulated by these proteins interactions, including those with histones [28–30]. VRK1 is also activated by DNA damage [31]. Therefore, VRK1 is a good candidate to participate in an early response mechanism to DNA damage. This mechanism detects and reacts to local alterations in chromatin structure induced by DNA damage. p53 is a transcription factor implicated in cellular responses to stress, including DNA damage [32]. In this context, it was proposed that p53 might be forming a complex with VRK1 in a basal readiness state [8]. VRK1 uniquely and specifically phosphorylates p53 in Thr–18 [7–9], leading to p53 stabilization and subsequent accumulation and initiation of p53 dependent transcription [8], which is consistent with this early role in damage response. The resulting p53 accumulation is reversed by a novel p53–dependent activation of autophagy that removes its activating VRK1 [33], a p53 stabilizer, and thus permits p53 dephosphorylation and its downregulation by mdm2 [9,15,33]. Moreover, this autoregulatory loop is altered in human cancers with p53 mutations [9,34]. Human VRK1 protein has also been implicated in the regulation of proliferation and cell cycle progression, where it plays several roles [35]. VRK1 is required for G0 exit, behaving like an early gene such as MYC and FOS [36]. VRK1 is also required for phosphorylation of histone H3 and chromatin compaction late in mitosis [27]. Moreover, VRK1 positively correlates with the proliferation phenotype in human head and neck [37] and lung cancers [34,38], and in breast cancer xenografts it also affects proliferation [39]. In addition, VRK1 kinase activity is enhanced in response to DNA damage and is an early participant necessary for the formation of 53BP1 foci in response to DNA double-strand breaks induced by ionizing radiation, in both resting and dividing cells [31]. In murine gene-trap models, VRK1 deficiency results in an early gene such as FOS and its downregulation by mdm2 [9,15,33]. Moreover, this autoregulatory loop is altered in human cancers with p53 mutations [9,34]. Human VRK1 protein has also been implicated in the regulation of proliferation and cell cycle progression, where it plays several roles [35]. VRK1 is required for G0 exit, behaving like an early gene such as MYC and FOS [36]. VRK1 is also required for phosphorylation of histone H3 and chromatin compaction late in mitosis [27]. Moreover, VRK1 positively correlates with the proliferation phenotype in human head and neck [37] and lung cancers [34,38], and in breast cancer xenografts it also affects proliferation [39]. In addition, VRK1 kinase activity is enhanced in response to DNA damage and is an early participant necessary for the formation of 53BP1 foci in response to DNA double-strand breaks induced by ionizing radiation, in both resting and dividing cells [31]. In murine gene-trap models, VRK1 deficiency results in sterility due to either meiotic defects in females or by affecting maintenance of spermatogonial stem cells prior to meiosis in males [40–43].

In this work we aimed to demonstrate the presence of a stable intracellular p53–VRK1 protein complex in non-damaged cells. We have determined that VRK1 activation in response to UV-induced DNA damage is accompanied by an immediate phosphorylation of p53 in Thr–18 within the complex, and that this early response precedes p53 accumulation in cells. These results indicate that the basal VRK1–p53 protein complex is a very early participant in DNA damage responses (DDR).

2. Materials and methods

2.1. Plasmids

Cloning of human full-length VRK1 (1–396) and VRK1ΔN (267–396) into mammalian expression vector pCEFL–GST have been described previously [44]. VRK1 mutants were generated using the Quick-Mutagenesis system (Stratagene, San Diego, CA) and specific primers (indicated in Table S1) following the manufacturer’s instructions and using plasmid pCEFL–HA–VRK1 as template. Plasmids expressing full-length human p53 and the mutants R273H, R248H and R280K were obtained from B. Vogelstein (John Hopkins University, Baltimore, USA) and we have used them previously [9,33]. Plasmids expressing GST–p53N-terminal transactivation domains for bacterial expression were obtained from D. Meek (University of Dundee, Scotland) and have been reported before [7] and human GST–p53 fusion proteins 1–390, 90–290 and 290–390 were from T. Kouzaridis (Cancer Research UK, Cambridge).

2.2. Antibodies

The p53 protein was detected with polyclonal antibody CM1 (Novocastra, Newcastle, UK) or monoclonal antibodies PAb-1801 and DO-1 (Santa Cruz, Santa Cruz, CA). Specific phosphorylation of p53 in Thr–18 was determined with a phosphospecific polyclonal antibody (Cell Signaling, Beverly, MA). VRK1 was detected with either rabbit polyclonal antibodies VC or VE or with monoclonal antibodies S6F6 or 1B5 [45]. Anti-myc polyclonal antibody (Upstate-Millipore, Temacula, CA) or anti-HA monoclonal antibody (Covance, Berkeley, CA) were either used for immunoprecipitation of transfected proteins or as negative control in endogenous immunoprecipitation assays. β-Actin was detected with the AC15 monoclonal antibody (Sigma, St. Louis, MO).

2.3. Cell lines, transfections, immunoprecipitation and pull-down assays

HEK293T, H1299 (p53–/–) and A549 cell lines were grown as monolayers in either DMEM or RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were detached with trypsin–EDTA solution. All culture reagents were from Invitrogen-GIBCO. In experiments in which plasmid constructs were transiently transfected, cells were in exponential growth phase [9,15]. Twenty-four hours before transfection cells were plated in P160 dishes at 60–70% confluence at the time of transfection as described previously [15,28], Pull-down and immunoprecipitation experiments were performed as reported previously [8,15]. Gels were transferred to Immobilon-P membranes (Millipore, Billerica, MA). Proteins in membranes were detected using a luminescence ECL kit (Amersham, GE Healthcare). Blots were quantified in the linear response range using an FX Personal Imager (Bio-Rad, Hercules, CA) [15].

2.4. Induction of DNA damage by UV light

DNA damage induced by UV-C light was performed with a Stratalinker (Stratagene, San Diego, CA). Cells were starved with media containing 0% FBS before treatment. The ultraviolet light (UV) dose delivered was determined by direct measurement with a radiometer Spectroline XS-254 nm-UVC (Spectronics Corporation, Westbury, N.Y.).

2.5. In vitro kinase assay

Endogenous VRK1 protein was immunoprecipitated with monoclonal antibody 1F6 as previously reported [44,45] and the immunoprecipitate was used for an in vitro kinase assay [46] showing either endogenous VRK1 autophosphorylation or phosphorylation of GST–p53 (1–85) used as substrate [7,46–48]. The kinase assay was performed at 30 °C for 30 min as previously described [47,48]. Samples were fractioned by SDS–PAGE and radioactivity was detected by exposure to X-ray film in the linear response range. Quantifications were done in a Personal Molecular Image FX (BioRad, Hercules, CA).
3. Results

3.1. VRK1 stably interacts with p53 forming a basal protein complex

Based on observations showing that VRK1 directly phosphorylates p53 in Thr-18 [7]; that its knockdown results in the loss of this phosphorylation [31,33] and that endogenous p53 and VRK1 proteins co-localize in nuclei of non-damaged [8], we asked whether p53 and VRK1 were able to form a stable protein complex. To detect this potential VRK1–p53 complex, reciprocal immunoprecipitation of endogenous proteins were performed in HEK293T cells and both proteins, VRK1 and p53, were detected (Fig. 1A), indicating that they are indeed forming a stable basal complex in non-damaged cells. Neither of them was detected in negative control with a non-specific antibody (Fig. 1A, bottom). The human VRK1 protein is characterized by a well-defined, but atypical, kinase domain comprised between residues 35 and 275 with an ATP binding site (residues 43–71) and a catalytic active site (residues 173–185) [7,16]. Kinase domains do not form stable complexes to permit release of its reaction products, ADP and the phosphorylated target protein. The VRK1 C-terminal region (residues 275–396) is characterized by its low complexity [49] and its flexibility [49]. This C-terminal region has been described to interact with several proteins and to play a regulatory role [28,29,31]. For these reasons, next we analysed if this VRK1 region was binding to p53 by performing pull-down assays and with two different GST-VRK1 constructs [44]; full-length VRK1 (residues 1–396) and VRK1ΔN comprising the C-terminus (residues 267–396) (Fig. 1B). We found that endogenous p53 interacted with both constructs suggesting that the C-terminus of VRK1 is the region involved in the binding. To further explore this interaction, several VRK1 phosphorylation mutants within the C-terminus (S342A, S342D, T355A, T355D, T378E, T390A and T390D) were also tested and found that none of them affected the VRK1–p53 interaction (Fig. S1). We also examined the effect of the double mutant VRK1 (E313G/K314I), a locally charged region of the VRK1 C-terminus, on the interaction (Fig. S2A and B) and we found that a change in the charge in this region resulted in increased binding. Noteworthy, when the mutation that causes spinal muscular atrophy, VRK1(R358X) [50], was tested we showed that VRK1 was still able to interact with p53.
(Fig. S2A and B). Finally, we analysed if the kinase activity of VRK1 was required for the binding. For this aim we carried out pull-down assays using wild-type VRK1 or kinase-dead VRK1 (K179E) (Fig. 1C). We found that the kinase-dead VRK1 interacted more strongly with p53, which is consistent with its inability to complete its catalytic cycle and indicates that the kinase activity is not necessary for an stable interaction between these two proteins, which is consistent with the structure of the folded protein [49]. In Fig. 1D is shown the suggested VRK1 region involved in the interaction with p53.

3.2. The p53 DNA binding domain (DBD) interacts with VRK1

The p53 protein has three well-defined structural and functional domains. The N-terminus trans-activation domain (TA) is involved in selecting its interaction with either ubiquitin ligases or with transcriptional cofactors, and its specificity is determined by the phosphorylation pattern. The DNA binding domain (DBD) is the p53 central region, and the C-terminus has and oligomerization (OD) and a regulatory subregions [51]. Next, we asked which of these p53 regions was implicated in its binding to VRK1. We performed pull-down assays using p53 fusion constructs expressing the different domains of p53 and endogenous VRK1 (Fig. 2A) and we found that VRK1 was able to bind only to full-length p53 (residues 1–390) and to its DNA binding domain (residues 90–290). Because VRK1 specifically phosphorylates p53 at Thr-18 within the TA domain [7], we also examined the binding of VRK1 to this p53 TA domain fused to GST that was expressed and purified in bacteria. In this pulldown, the N-terminus of p53 did not interact with VRK1 (Fig. 2B). Therefore, we conclude that the N- and C-terminal domains of p53 do not participate in the stable interaction with VRK1. In Fig. 2C is depicted the suggested interacting region of p53 with VRK1.

3.3. p53 DNA contact mutations do not disrupt its interaction with VRK1

The TP53 gene is frequently mutated in many types of cancer, and most of these mutations occurred within the DBD [52]. Because we identified that VRK1 binds to p53 through this region we analysed whether some of these common p53 mutations [53] affected its binding to VRK1. For this aim we performed pull-down assays using transfected HEK293T cells with GST–VRK1 and different plasmids expressing human p53 mutants including the conformational mutant R175H and DNA-contact mutants such as R248W, R273H and R280K (Fig. 3A). These results were confirmed by immunoprecipitation of endogenous VRK1 in H1299 cells transfected with wild-type p53, R175H and R248W (Fig. 3B). We found that all mutant proteins stably interacted with VRK1; however, the interaction with the conformational mutant R175H was
approximately four times stronger compared to control wild-type or the contact-DNA mutant R248W. These findings also indicate that the interaction of VRK1 with p53 in the DBD occurs by the globular region that is not directly interacting with DNA grooves.

3.4. The basal VRK1–p53 complex is immediately activated in response to UV light treatment

VRK1 kinase activity is regulated by the folding of its C-terminal region [29,49] that results in a conformational change in native protein that is recognized by a specific monoclonal antibody (1B5) [45]. Previous work has shown this structural change in cells upon DNA damage response to ionizing radiation [31]. Because we have detected a stable VRK1–p53 complex in non-damage cells next we asked whether this complex could be activated in response to DNA damage and consequently have an effect on both proteins. Therefore, we determined if this conformational change was detected in nuclear VRK1 after induction of DNA damage by UV-light (Fig. S3A). We found that in UV-treated cells there was a 30% increase in VRK1 nuclear staining detected with the 1B5 monoclonal antibody by immunofluorescence. This increase in reactivity reached its maximum value at short times, five to ten minutes, after UV-irradiation. No variation in VRK1 nuclear protein reactivity was observed using a polyclonal antibody that does not detect the conformational change (Fig. S3A). VRK1 protein levels are not affected by UV-light treatment as shown by immunoblotting analysis using the 1B5 antibody to detect the denatured VRK1 protein (Fig. S3B). Based on this initial observation we proceeded to examine if the kinase activity of VRK1 was enhanced upon UV-light treatment. For this aim we carried out an in vitro kinase assay with endogenous VRK1 protein immunoprecipitated from HEK293T cells treated or not with UV-light and GST–p53.
(residues 1–85) used as substrate (Fig. 4A). We found that UV-light treatment increased between 6 and 12-fold the level of both VRK1 autophosphorylation (activation) and p53 phosphorylation (Fig 4A, graph). This observation led us to investigate if VRK1 activation could also result in an increase in p53 phosphorylation at Thr-18 after UV-induced DNA damage. For this assay, we focused on the endogenous p53 protein that is forming part of the basal VRK1–p53 complex. For this aim HEK293T cells were treated with UV light and a time course was performed to measure the specific phosphorylation of p53 in Thr-18 present in the immunoprecipitated complex (Fig. 4B). We found that the VRK1–p53 complex is already detected in non-treated cells. At short times after UV treatment, the protein levels of both VRK1 and p53 remained constant, but there is an increase in p53-Thr18 phosphorylation in the p53 immunoprecipitated complex that also contain VRK1 (Fig. 4B). This immediate accumulation of phosphorylated p53 in Thr-18 was quantified (Fig. 4B, graph). These results are consistent with the loss of p53-Thr18 phosphorylation detected in UV-treated [33], or treated with ionizing radiation [31], in which VRK1 was knocked down. These findings indicate that there is an early p53 switch towards its transcriptional activation mode as a consequence of its immediate phosphorylation by VRK1 in Thr-18 that is inducible by DNA damage and precedes p53 accumulation. Thus we conclude that specific phosphorylation of p53 at Thr-18 is an immediate event occurring in the pre-existing basal p53–VRK1 complex during the cellular response to UV-induced DNA damage. In Fig. 4C is shown the proposed model of the VRK1–p53 complex dynamics upon UV-induced DNA damage.

3.5. VRK1 is integrated in DNA-damage response pathways

The direct interaction and phosphorylation of p53 by VRK1 and other proteins implicated in DNA damage responses led us to start the elucidation of the early steps in the signalling pathway

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**Fig. 4.** UV-induced activation of VRK1 and phosphorylation of p53 in the VRK1–p53 complex. (A) Activation of endogenous VRK1 by UV-induced DNA damage. Serum deprived HEK293T cells were treated or not with UV-C light in the absence of serum to reduce VRK1 activation by growth factors. The endogenous VRK1 protein present in the lysate (bottom panel) was immunoprecipitated with antibody 1F6 as reported previously [44,45] and the immunoprecipitated protein was used for an in vitro kinase assay with ATP-[32P] showing either endogenous VRK1 autophosphorylation or phosphorylation of GST–p53 (1–85) [7,46]. The top panel shows the activation (autophosphorylation) of VRK1 and the level of protein immunoprecipitated. The middle panel shows the phosphorylation of p53 and the total p53 protein present in the assay. The ratio of phosphorylated to total protein was quantified in the linear response range and is represented in the graph (right). (B) Effect of UV-induced damage on p53-Thr18 phosphorylation complexed with VRK1. Serum deprived HEK293T cells were irradiated with UV-C light and endogenous p53 was immunoprecipitated at different time points. The level of Thr18-phosphorylation was determined with a phosphospecific antibody. In the p53 immunoprecipitate, the presence of endogenous VRK1 was also determined. The amount of p53, p53-Thr18-P and VRK1 proteins was quantified in the linear response range and is represented in the graph (right). The control point (0 min) was immunoprecipitated either with a negative control antibody as well as with anti-p53. (C) Model illustrating the dynamics of the VRK1–p53 complex upon DNA damage in cells. In non-damaged cells, VRK1 and p53 form a stable complex ready to respond to injury (left). Upon DNA damage, VRK1 gets activated (autophosphorylation) and in turn activates p53 by phosphorylation at Thr-18, which precedes its accumulation (right).
responding to DNA damage. In this context, VRK1 is highly likely to play a coordinating role among the different DDR signalling components and sequential processes, which range from damage detection to assembly of specific repair systems and finally restoration to normal chromatin structure. Thus, the proteins interacting with VRK1 were analysed using the Intact database with the Cytoscape software [54] that outlined the initial components of the VRK1 pathway in the context of proteins associated to DNA damage responses and transcription factors (Fig. 5). The formation of a complex between accumulated p53 mutants and VRK1 can result in sequestration of VRK1 and have an effect on VRK1 dependent functions.

4. Discussion

Any DNA structural alteration must be detected as a locally altered chromatin, which should activate chromatin kinases and initiate a complex process aiming to achieve the full reparation of damaged DNA. This process requires several sequential steps implicating different components [55,56]. Mechanistically, local distortion of chromatin as a consequence of DNA damage should alter proteins directly interacting with histones. In this context VRK1, which is a nucleosomal histone kinase whose activity is regulated by histone interactions [27,29], is a very likely participant in sensing and initiating the early response to DNA damage by regulation of transcription factors implicated in DDR, such as p53 [8,33] or c-Jun [25], since both are targeted by VRK1. The basal interaction between wild-type p53 and VRK1 forming a stable complex in non-damaged cell suggests a well-defined sensing role for this protein complex in DNA-damage responses. The main role of the VRK1–p53 complex is a fast initial response by sensing the locally altered chromatin structure that activates VRK1 to phosphorylate p53 in Thr18 [7], which switches p53 to a transcription factor binding mode [11,13], triggering p53-dependent DDR. The detection of this VRK1–p53 protein basal complex is also consistent with one of the roles of VRK1 within the nucleus, the formation of VRK1 complexes by direct interactions with transcription factors that are gradually regulated by p53 activation [13,14]. Thus, formation of a protein complex between VRK1 and p53 adds another transcription factor to those that are already known to stably interact with VRK1, which can have functionally different consequences based either on the interaction with p53, wild-type or mutated, and on protein levels in individual cell types. The pre-existing complex formed by VRK1–p53 suggests a very early role for VRK1 in DNA damage-responses (DDR), and is also consistent with previous observations in which VRK1 knockdown prevented the activating phosphorylation of ATM, CHK2 and DNA-PK, as well as p53-Thr18 phosphorylation, in response to ionizing radiation [31]. The formation of a VRK1–p53 complex can have additional effects based on the roles that are performed by each individual participating protein. p53 mutations in the DNA binding domain are unable to induce their own downregulation mediated by gene expression of HDM2/MDM2 coding for an ubiquitin ligase and resulting in p53 mutant accumulation. Moreover, this defective induction of gene expression by p53 also affects downregulation of VRK1 protein [33]. VRK1 downregulation is mediated by p53 transcriptional activation of DRAM (damage-regulated autophagic modulator) [57]. DRAM targets VRK1 to enter in the autophagic pathway [33] and thus be degraded in the lysosome [9,15]. This autoregulatory loop is altered in tumours with p53 mutations [34], resulting in an accumulation of VRK1 protein, which has been detected in human lung [34] and head and neck squamous cell carcinomas [37].

The situation in tumours harbouring p53 mutations is very likely to be functionally different [58]. Mutant p53 proteins accumulate in cells and can be targeted by kinases, and be also able to interact with and sequester other cellular proteins. Thus, p53 mutant direct interaction with other cellular proteins is very likely to alter their normal function. The differential interaction among p53 mutants and VRK1 can contribute to partly explain their differential effects. Differences in biological effects have already been associated to different p53 mutations [53,59,60], which may also be dependent on cell type and its interactome. The p53 mutants R175H and R273H appear to facilitate cell invasion by interaction...
with the NRD1 metalloprotease [61], or by modulating MET signalling [62]. Also p53 mutants, by their incorporation in gene promoters containing the SREBP transcription factor can regulate genes in the mevalonate pathway [63]. Furthermore, p53 mutant proteins can also bind to other cellular proteins and interfere with their function, such as the Mre11 complex [64]. High levels of mutant p53 proteins can also interact with transcription factors [65] or transcriptional co-activators. Therefore, p53 mutants can be integrated in different gene promoters and might alter their normal functions [59,66].

In addition the accumulation of p53 mutants can also sequester VRK1, and consequently the formation of a protein complex between mutated p53 and VRK1 might result in the formation of protein complex that might interfere with other VRK1 dependent functions, such as VRK1 responses to DNA damage [31,33]. In addition, mutated p53 can sequester VRK1 from its participation in other transcriptional complexes, such as those between VRK1 and Jun [25], ATF2 [26] or CREB [24] and therefore might affect other transcriptional complexes, such as those between mutated p53 and VRK1 might result in the formation of protein complexes with BIP/p90 and HDM2. Oncogene 28, 2112–2118.


