

Report

Pro-apoptotic function of checkpoint kinase-2 in syncytia elicited by the HIV-1 envelope

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Abbreviations: AIDS, acquired immunodeficiency syndrome; ATM, ataxia telangiectasia mutated; ATMS1981P, ATM phosphorylated on serine 1981; Cdk1, cyclin-dependent kinase 1; Chk1, checkpoint kinase-1; Chk1S317P, Chk1 phosphorylated on serine 317; Chk2, checkpoint kinase-2; Chk2T68P, Chk2 phosphorylated on threonine 68; Cyt. *c*, cytochrome *c*; Env, envelope glycoprotein complex; GAPDH, glyceraldehyde phosphate dehydrogenase; γ -H2AX, phosphorylated histone 2AX; HIV-1, human immunodeficiency virus-1; KG, karyogamy; MAPK, mitogen activated protein kinase; MOMP, mitochondrial outer membrane permeabilization; NBS1, nijmegen breakage syndrome 1; PML, promyelomonocytic leukemia; Pre-KG, pre-karyogamy; SC, single cell; siRNA, small interfering RNA; Syn, syncytia; TopBP1, topoisomerase II β -binding protein 1

Key words: AIDS, apoptosis, ATM, DNA damage, envelope, PML

Fusogenic HIV-1 isolates induce the fusion of infected and bystander cells. Such syncytia can be found as “multinucleated giant cells” in the brain from HIV-1-infected individuals, as well as in lymphoid tissues. Syncytia elicited by the HIV-1 envelope glycoprotein (Env) manifest the aggregation of PML in discrete nuclear bodies and the recruitment of TopBP1, NBS1 and ATM to DNA damage foci containing phosphorylated ATM and histone H2AX (γ -H2AX). This DNA damage response then culminates in p53-dependent activation of the mitochondrial pathway of apoptosis. Here, we show that Env-elicited syncytia also manifest activating phosphorylations of the checkpoint kinases 1 and 2 (Chk1 and Chk2), and both Chk1 and Chk2 colocalize with γ -H2AX foci. However, only the siRNA-mediated knockdown of Chk2, not the depletion of Chk1, inhibits mitochondrial outer membrane permeabilization and subsequent syncytial apoptosis. Depletion of PML, TopBP1, NBS1 or ATM inhibit the activating phosphorylation of Chk2. Altogether, these results indicate that Chk2 (but not Chk1) participates in the DNA damage-elicited pro-apoptotic cascade that leads to the demise of Env-elicited syncytia.

Introduction

Human immunodeficiency virus (HIV), the agent that causes acquired immunodeficiency syndrome (AIDS), triggers the apoptotic

demise of HIV-1-infected cells (“direct killing”) as well as the destruction of noninfected cells (“bystander killing”).¹⁻⁴ Several HIV-1 gene products have apoptogenic properties. Among these gene products, the envelope glycoprotein complex (Env) is particularly important, because viruses in which Env has been replaced by the envelope of vesicular stomatitis virus lose much of their apoptosis-driving potential.^{5,6} The membrane-anchored Env gp120/gp41 complex exposed on the plasma membrane surface of HIV-1-infected cells can trigger apoptosis through an interaction with uninfected cells expressing the Env receptor (CD4) and the chemokine co-receptor (CXCR4 or CCR5).⁷⁻¹⁰ This type of bystander killing often involves cell-to-cell fusion events that lead to the formation of syncytia. Such syncytia are relatively rare in lymphoid tissues from HIV-1-infected patients,^{11,12} perhaps due to their short half-life. However, syncytia dubbed as “giant multinuclear cells” are pathognomonic for the diagnosis of HIV-1-associated encephalitis (also called “neuro-AIDS”), a neurodegenerative condition that leads to dementia.¹³⁻¹⁶

In recent years, we have taken advantage of an in vitro model of syncytium formation to elucidate the pro-apoptotic signal transduction pathway elicited by HIV-1-encoded Env. The initial fusion of the plasma membrane of Env-expressing and CD4/CXCR4-expressing cells causes the accumulation of several nuclei within one single cytoplasm (cytogamy). After a latency, non-synchronized nuclei fuse as a result of the Cdk1/cyclinB-mediated destruction of the nuclear envelope (karyogamy)^{7,17,18} and then manifest a DNA damage response, as indicated by the presence of phosphorylated ATM that co-localizes with its substrate, phosphorylated histone H2AX (γ H2AX) in discrete foci.¹⁹ This DNA damage response likewise can be attributed to the karyogamic juxtaposition of chromosomes that are in distinct phases of the cell cycle, including the S and the G₂/M phases.¹⁹ Upstream of ATM, PML aggregates

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Submitted: 12/10/08; Revised: 12/12/08; Accepted: 12/15/08

Previously published online as a *Cell Cycle* E-publication:
<http://www.landesbioscience.com/journals/cc/article/7642>

in discrete nuclear bodies that partially co-localize with TopBP1 and NBS1 as well as phosphorylated ATM.²⁰ Downstream of ATM, p38 MAPK leads to the activating phosphorylation of p53 on serines 15 and 46, thereby inducing a transcriptional program that culminates in the overexpression of pro-apoptotic proteins of the Bcl-2 family (such as Puma and Bax),^{17,21-23} followed by Bax-dependent mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release, caspase activation and apoptotic cell death.^{24,25} Importantly, multiple elements of this cascade (Cdk1 phosphorylation, PML aggregation, phosphorylation of ATM, H2AX, p38 and p53 and overexpression of Puma) have been detected by immunohistochemical methods within giant multinuclear cells from the brain of patients with HIV-1-associated encephalitis, as well as in syncytia present in the lymph node from therapy-naïve HIV-1 carriers.^{17,19-21,23,26}

During the DNA damage response, the checkpoint kinases-1 and -2 (Chk1/2) are activated by ATR and ATM, respectively,^{27,28} although some crosstalk among these pathways occurs.²⁹ Activating phosphorylations of Chk1 and Chk2 can be detected in tetraploid cancer cell lines³⁰ and in polyploid syncytia,^{31,32} respectively. However the molecular etiology of these activating phosphorylations is not understood in detail. We therefore decided to study the relative contribution of Chk1 and Chk2 to the apoptosis of Env-elicited syncytia. Here, we show that Chk2 (but not Chk1) contributes to syncytial apoptosis.

Results and Discussion

Activating phosphorylation of Chk1 and Chk2 in Env-elicited syncytia. When HeLa cells stably transfected with the HIV1^{LAI/IIIIB} *env* gene are cocultured with HeLa cells stably transfected with *CD4* (expressed together with endogenous CXCR4), the two cell types rapidly form syncytia, which after an initial phase of pure cytogamy (or pre-karyogamy) undergo karyogamy (nuclear fusion). Immunoblot analyses and confocal immunofluorescence microscopy with antibodies specific for phospho-neoepitopes, revealed that both Chk1 and Chk2 underwent activating phosphorylations on serine 317 (Chk1S317P) and threonine 68 (Chk2T68P), respectively. These activating phosphorylations were restricted to the karyogamic stage of syncytia and were near-to-undetectable in single cells and pre-karyogamic syncytia (Fig. 1A–F). The vast majority of karyogamic syncytia (>70%) as well as most syncytia that had become apoptotic, after karyogamy, stained positively for Chk1S317P and Chk2T68P. Phosphorylated Chk1 and Chk2 were found within the nuclei, in a speckled pattern that co-localized with γ -H2AX-positive foci (Fig. 1B and E), suggesting that the activation of both kinases is tightly linked to the DNA damage response.

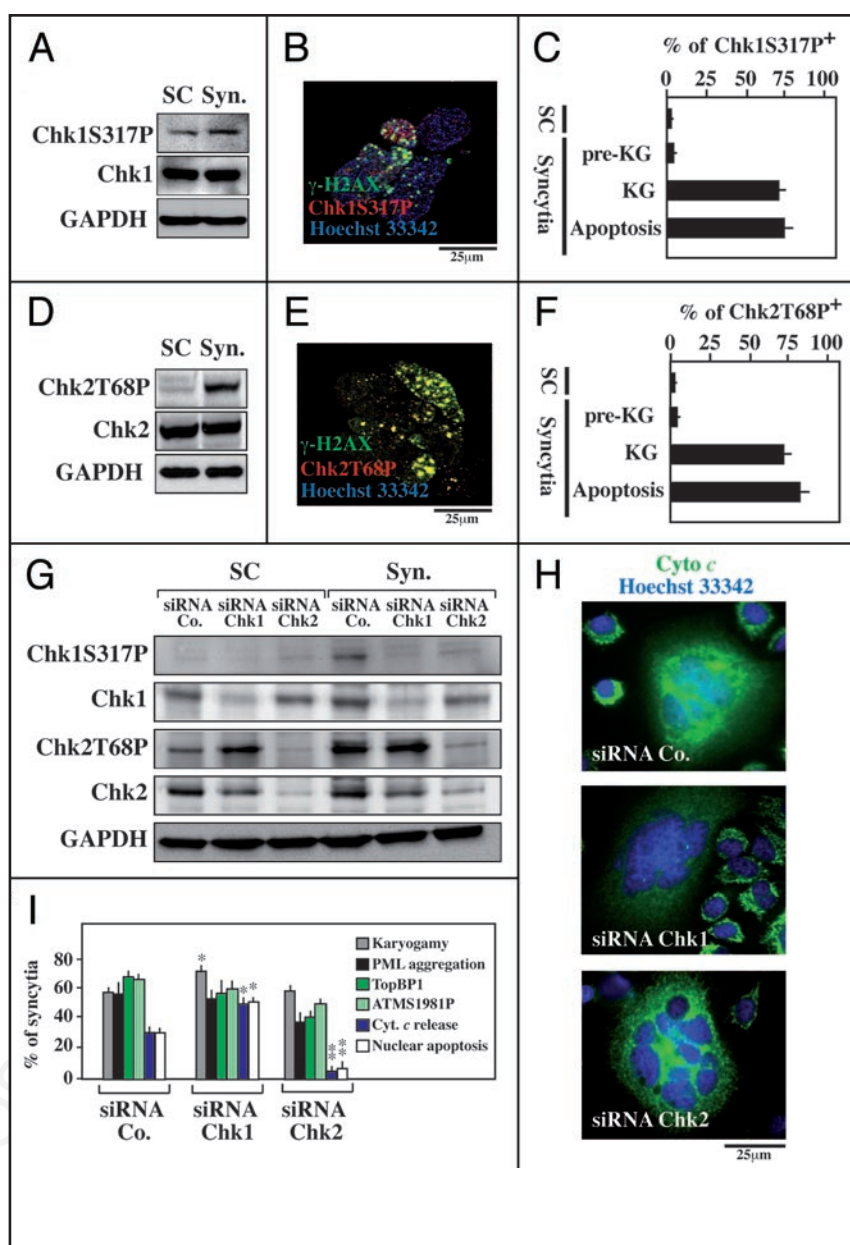


Figure 1. Contribution of Chk1 and Chk2 to syncytial apoptosis. (A and D) Chk1S317P, Chk2T68P, Chk1 and Chk2 expression levels. Extemporaneous 1:1 mixtures of HeLa Env and HeLa CD4 single cells (SC) or syncytia (48 h) were subjected to immunoblot detection of Chk1S317P, Chk2T68P, Chk1, Chk2 and GAPDH (A and D). (B and E) Karyogamic syncytia reveal Chk1S317P⁺ (B) and Chk2T68P⁺ foci (E) at sites of DNA strand breaks (as detected by H2AXS139P). Representative immunofluorescence pictures of karyogamic syncytia are shown after staining with anti-Chk1S317P (B), anti-Chk2T68P (E) and anti-H2AXS139P (B and E). (C and F) Frequency of single cell (SC), pre-karyogamic (PreKG), karyogamic (KG) and apoptotic syncytia showing foci containing Chk1S317P (C) or Chk2T68P (F). (G) Effects of Chk1- or Chk2-knockdown on Chk1S317P, Chk1, Chk2T68P, Chk2 and GAPDH expression levels. (H and I) Effects of the downregulation of Chk1 or Chk2 expression on syncytial apoptosis. HeLa Env and HeLa CD4 cells were transfected during 48 hours with small interfering RNA against Chk1 or Chk2. Syncytia arising from the coculture of these transfected cells were stained for the detection of karyogamy, PML aggregation, TopBP1⁺ foci formation, ATMS1981P, Cyto *c* release and nuclear apoptosis. Representative immunofluorescence pictures revealing consequences of Chk1- or Chk2-knockdown on cytochrome *c* release of karyogamic syncytia (H). Note that Chk1-knockdown increases syncytial apoptosis and that Chk2-knockdown reduces this cell death. Karyogamy is determined by staining with Hoechst 33342 and fluorescence microscopy. **p* < 0.01 and ***p* < 0.001 as compared to siRNA-transfected control.

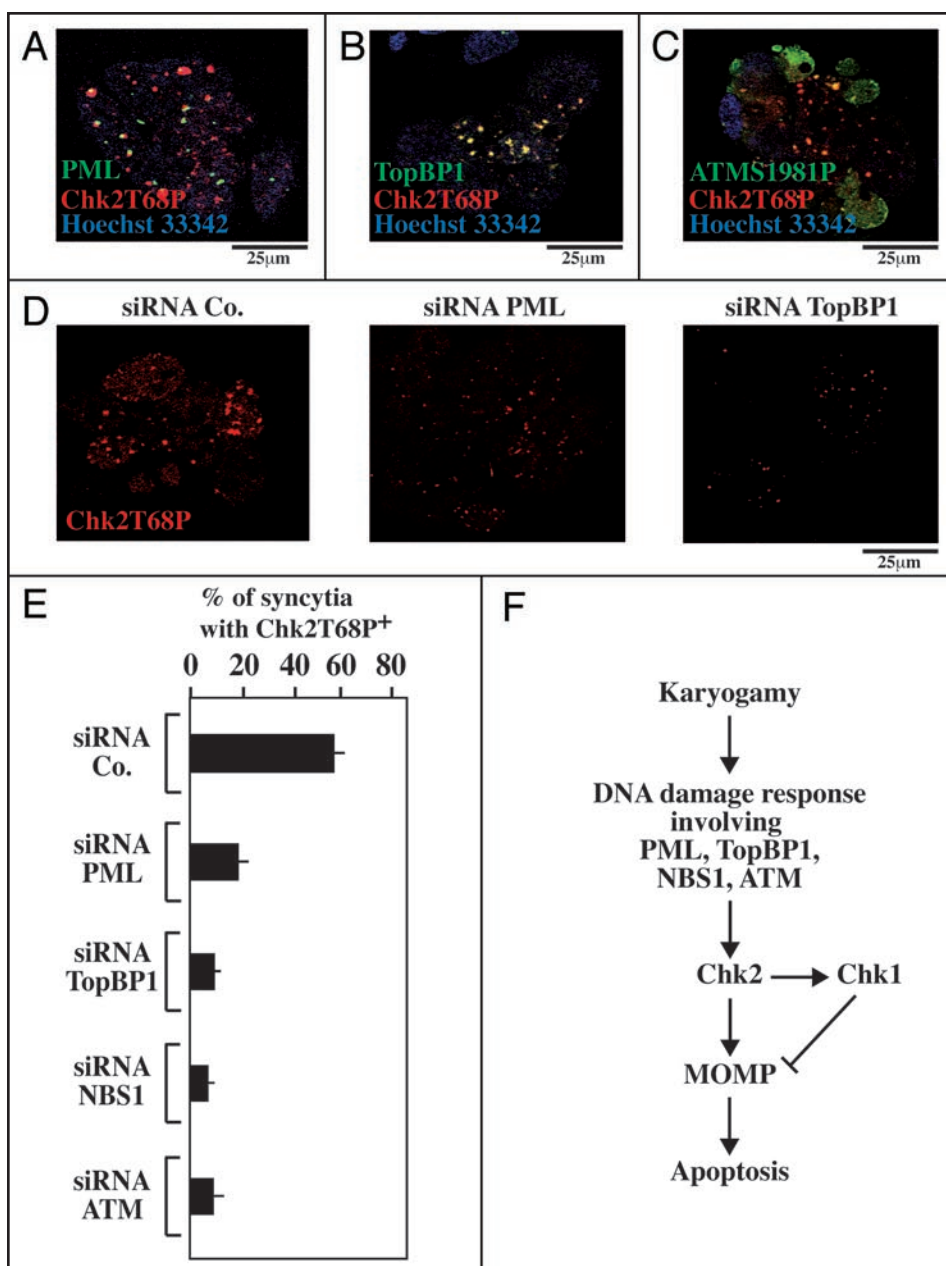


Figure 2. Phosphorylation of Chk2 on threonine 68 is controlled by PML, TopBP1, NBS1 and ATM. (A–C) Coimmunolocalisation of Chk2T68P with PML⁺, TopBP1⁺ or ATMS1981P⁺ foci. Notes that Chk2T68P colocalizes partially with PML (A), TopBP1 (B) and ATMS1981P (C) on karyogamic syncytia. (D and E) Effects of PML-, TopBP1-, NBS1- and ATM-knockdown on Chk2T68P⁺ foci formation. HeLa Env and HeLa CD4 were transfected with small interfering RNA against PML, TopBP1, NBS1 and ATM during 48 hours. Then, syncytia are generated by coculture for 48 h. Representative immunofluorescence photographs show that PML or TopBP1 downregulations reduce the percentage of syncytia containing Chk2T68P⁺ foci (D). Knockdown of PML, TopBP1, NBS1 and ATM by small interfering RNA reduce the frequency of syncytia revealing Chk2T68P⁺ foci (E). (F) Hypothetical involvement of Chk1 and Chk2 in the lethal signal transduction cascade leading to syncytial apoptosis.

Differential implication of Chk1 and Chk2 in syncytial apoptosis. Next, we evaluated the effects of the knockdown of either Chk1 and Chk2 with specific small interfering RNAs (siRNAs) (Fig. 1G). While the knockdown of Chk1 did not affect the activating phosphorylation of Chk2, the depletion of Chk2 did reduce the phosphorylation of Chk1, suggesting that Chk2 might operate upstream of Chk1 (Fig. 1G). The knockdown of Chk2 (but not

that of Chk1) caused a drastic reduction in MOMP and apoptosis. Thus, approximately 25% of syncytia that had been generated from cells transfected with a control siRNA manifested the release of cytochrome *c* from mitochondria and underwent nuclear chromatin condensation as a sign of apoptosis, 48 h after initiation of the coculture (Fig. 1H and I). However, less than 10% of the syncytia depleted from Chk2 manifested signs of MOMP and apoptosis (Fig. 1H and I). In contrast, the knockdown of Chk2 did not affect the aggregation of PML and of TopBP1 nor the activating phosphorylation of ATM on serine 1981 (ATMS1981P), in line with the idea that Chk2 operates downstream of the DNA damage response but upstream of the final apoptotic pathway. The knockdown of Chk1 had rather different effects; it had no effect on PML or TopBP1 aggregation, did not affect ATMS1981P, yet caused a major increase in MOMP and apoptosis that both affected approximately 50% of Chk1-depleted syncytia (Fig. 1H and I). Altogether, these results indicate that Chk2 is a pro-apoptotic signal transducer in syncytia while Chk1 has an antiapoptotic effect.

Chk2 activation downstream of PML, TopBP1, NBS1 and ATM. To further investigate the hierarchical relationship between the activating Chk2 phosphorylation and upstream mediators of the DNA damage response, we investigated the co-localization of Chk2T68P and PML, TopBP1 or ATMS1981P by confocal immunofluorescence, in karyogamic syncytia (Fig. 2A–C). In all cases, we found a partial colocalization of Chk2T68P with PML, TopBP1 or ATMS1981P, pointing to a functional relationship (Fig. 2A–C). Indeed, the knockdown of PML, TopBP1, NBS1 or ATM caused a major decrease in the Chk2 phosphorylation within karyogamic syncytia (Fig. 2D and E), confirming that Chk2 is indeed activated as a result of the DNA damage response.

Materials and Methods

Cell lines, cell culture and antibodies.

HeLa cells stably transfected with the *env* gene of HIV-1_{LAI/IIIB} (HeLa Env) and HeLa cells transfected with *CD4* (HeLa CD4) were cultured at a 1:1 ratio in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin (Invitrogen). Monoclonal antibodies against ATM, ATMS1981P, H2AX and H2AXS139P (γ -H2AX) were purchased from Upstate. Monoclonal anti-GAPDH antibody was obtained from Chemicon. Polyclonal rabbit or sheep

antibodies from Cell Signaling Technology were used for detection of Chk1, Chk1S317P, Chk2, Chk2T68P. The monoclonal anti-cytochrome *c* and anti-TopBP1 were obtained from Becton Dickinson.

Transfection and RNA interference. The knockdown of ATM (CAU ACU ACU CAA AGA CAU UdTdT),³⁹ Chk1 (GCG UGC CGU AGA CUG UCC AdTdT),⁴⁰ Chk2 (UGU GUG AAU GAC AAC UAC UdTdT),⁴⁰ NBS1 (CAG GAG GAA GAT GTC AAT GdTdT),¹⁹ PML (CUC CCA GGG ACC CUA UUG AdTdT),²⁰ or TopBP1 (CUC ACC UUA UUG CAG GAG AdTdT),²⁰ was achieved after transfection with Oligofectamine 2000 (Invitrogen).

Immunofluorescence. Cocultures were fixed with paraformaldehyde (4% w:v), permeabilized with 0.1% SDS and incubated during 20 minutes with FCS. Rabbit polyclonal antibodies specific for Chk1S317P and Chk2T68P were revealed with a goat anti-rabbit IgG conjugated to Alexa 568 (red fluorescence) from Invitrogen. Cells were also stained for the detection of ATMS139P, cytochrome *c*, H2AXS139P, PML, TopBP1 and revealed with an anti-mouse IgG conjugated to Alexa 488 (green fluorescence) from Invitrogen. To evaluate karyogamy and apoptotic chromatin condensation, cells were counterstained with Hoechst 33342 (Invitrogen) as previously described.^{19,20} Immunofluorescence images were obtained using an LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany).

Immunoblot. Whole cell protein lysates were extracted in lysis buffer (250 mM NaCl, 0.1% NP40, 5 mM EDTA 10 mM Na₃VO₄, 10 mM NaF, 5 mM DTT, 3 mM Na₄P₂O₇ and protease inhibitor cocktail from Roche). After brief sonication and centrifugation, proteins quantification was performed according to standard procedures. Aliquots of protein extracts (20 µg) were separated by 3–8% SDS-PAGE, transferred to nitrocellulose membrane at 4°C, blocked in 5% bovine serum albumin for 2 h with buffer and incubated during overnight with antibodies against Chk1, Chk1S317P, Chk2, Chk2T68P and GAPDH at 4°C. Then, the second appropriate antibodies (goat anti-rabbit, goat anti-sheep or goat anti-mouse; Southern Biotechnology) conjugated to horseradish peroxidase were incubated during 1 h and revealed by means of the enhanced ECL chemiluminescence detection system.

Concluding Remarks

The results reported in this article support the general contention that elements of the DNA damage response pathway are essential for the apoptotic demise of Env-elicited syncytia. In the past, we have found that knockdown or chemical inhibition of ATM inhibited syncytial apoptosis, while inhibition of ATR had no such effect.¹⁹ In apparent accord with the fact that ATM transduces its downstream effects mainly via Chk2 (while ATR acts mainly via Chk1),^{33–35} we describe here that inhibition of Chk2 (but not that of Chk1) interrupts the pro-apoptotic signal transduction cascade that operates in karyogamic syncytia (Fig. 2F). At first glance, this may appear in contradiction with previous reports from our group showing that a dominant-negative mutant of Chk2 or chemical inhibitors of Chk2, debromohymenialdisine and 2-(4-(4-Chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide, augmented the apoptotic demise of syncytia, secondary to the leverage of a mitotic block.^{20,31} However, it is well possible that the dominant-negative Chk2 mutant as well as the chemical inhibitors affect the function of other kinases than Chk2 (including Chk1 and perhaps others), and that their impact on syncytial apoptosis thus must be ascribed to off-target effects.

Paradoxically, we found that siRNA-mediated knockdown of Chk1 increased syncytial apoptosis downstream of the DNA damage response. We have recently found that specific inhibitors of Chk1, as well as the siRNA-mediated depletion of Chk1 can also trigger the death of tetraploid cancer cell lines in conditions in which Chk1 inhibition/depletion barely affects the survival. This suggests that the inhibition of Chk1 maybe a general strategy to eliminate tetraploid and higher-order polyploidy cells, irrespective of whether they have been generated by mitotic slippage or by cell fusion. Paradoxically, the apoptosis-inhibitory activation of Chk1 requires that of Chk2, suggesting that Chk2 cannot only trigger the apoptotic pathway (including the transactivation function of p53),^{29,36} but that Chk2 can also activate—through Chk1—an anti-apoptotic pathway. How this effect is achieved in molecular terms, for instance by inhibition of caspase-2 activation,³⁷ remains to be studied.

At present, it remains an open conundrum how exactly the DNA damage response that causes the pro-apoptotic activation of Chk2 (and the anti-apoptotic activation of Chk1) is activated in Env-elicited syncytia. It has been shown that the integration of the HIV-1 genome into host chromosomes involves the induction of DNA lesions that lead to the activation of ATM and that rely on ATM-dependent repair processes to be resolved.³⁸ However, the experimental system used here was virus-free, meaning that other mechanisms must come into action. We speculate that illicit cell fusion that affects proliferating, asynchronous cell populations, leads to a mixture of completely and partially replicated genomes stimulating a massive DNA damage response. However, the molecular details of this pathway are elusive.

Irrespective of these incognita, it appears clear that Chk1 and Chk2 emerge as important signal transducers in syncytial apoptosis and that modulation of either of these two druggable checkpoint kinases might have a profound impact on AIDS development.

Acknowledgements

GK is supported by Ligue contre le Cancer (équipe labellisée), Agence Nationale pour la Recherche (ANR), Agence Nationale pour la Recherche sur le SIDA (ANRS), Cancéropôle Ile-de-France, European Commission (Active p53, Apo-Sys, Chemores, TransDeath, Right, Death-Train), Fondation pour la Recherche Médicale, Institut National du Cancer (INCa), and Sidaction.

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