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Site-specific hyperphosphorylation of pRb in HIV-induced neurotoxicity ☆

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ABSTRACT

HIV-Associated Neurocognitive Disorder (HAND) remains a serious complication of HIV infection, despite combined Anti-Retroviral Therapy (cART). Neuronal dysfunction and death are attributed to soluble factors released from activated and/or HIV-infected macrophages. Most of these factors affect the cell cycle machinery, determining cellular outcomes even in the absence of cell division. One of the earliest events in cell cycle activation is hyperphosphorylation of the retinoblastoma protein, pRb (ppRb). We and others have previously shown increased ppRb expression in the CNS of patients with HIV encephalitis (HIVE) and in neurons in an *in vitro* model of HIV-induced neurodegeneration. However, trophic factors also lead to an increase in neuronal ppRb with an absence of cell death, suggesting that, depending on the stimulus, hyperphosphorylation of pRb can have different outcomes on neuronal fate. pRb has multiple serines and threonines targeted for phosphorylation by distinct kinases, and we hypothesized that different stimuli may target separate sites for phosphorylation. Thus, to determine whether pRb is differentially phosphorylated in response to different stimuli and whether any of these sites is preferentially phosphorylated in association with HIV-induced neurotoxicity, we treated primary rat mixed cortical cultures with trophic factors, BDNF or RANTES, or with the neurotoxic factor, N-methyl-D-aspartate (NMDA), or with supernatants containing factors secreted by HIV-infected monocyte-derived macrophages (HIV-MDM), our *in vitro* model of HIV-induced neurodegeneration. We found that, while BDNF and RANTES phosphorylated serine807/811 and serine608 *in vitro*, treatment with HIV-MDM did not, even though these trophic factors are components of HIV-MDM. Rather, HIV-MDM targets a specific phosphorylation site, serine795, of pRb for phosphorylation *in vitro* and this ppRb isoform is also increased in HIV-infected brains *in vivo*. Further, overexpression of a nonphosphorylatable pRb (ppRb S795A) attenuated HIV-MDM-induced neurotoxicity. These findings indicate that HIV-infection in the brain is associated with site-specific hyperphosphorylation of pRb at serine795, which is not induced by other tested stimuli, and that this phosphorylation contributes to neuronal death in this disease, demonstrating that specific pRb sites are differentially targeted and may have diverse impacts on the viability of post-mitotic neurons.

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Introduction

In the Central Nervous System (CNS), HIV manifests itself clinically as HIV-Associated Neurocognitive Disorder (HAND), which is estimated to affect 50% of the HIV(+) population in the current era of combined Anti-Retroviral Therapy (cART) (Dore et al., 1999; Heaton et al., 2010; Masliah et al., 2000; McArthur, 2004; Neuenburg et al., 2002). The neurological manifestations of HAND encompass a multitude of cognitive, behavioral, and motor deficits of varying severity (McArthur, 2004; Navia et al., 1986; Portegies et al., 1993; Woods et al., 2009). While the incidence of the most severe forms of HAND has decreased significantly with the widespread use of cART, subtler forms of HAND have become more prevalent (Heaton et al., 2010). Interestingly, there is little evidence supporting direct infection of neurons by HIV. Rather, culminating data support a model whereby HIV crosses the blood–brain barrier *via* infected monocytes that differentiate into macrophages and establishes a persistent infection within macrophages/microglia (M/M), creating a viral reservoir within the brain (Gendelman et al., 1994; Gonzalez-Scarano and Martin-Garcia, 2005a). These infected/activated M/M alter the extracellular environment by releasing numerous soluble factors, including viral proteins, cytokines, chemokines, quinolinic acid, TNF-α, and reactive oxygen species, which results in release of more of these and other neurotoxic and pro-inflammatory factors from astrocytes, neurons, and microglia (Giulian et al., 1996; Power and Johnson, 1995; Price et al., 1988; Soontornniyomkij et al., 1998). On the other hand, non-toxic trophic factors are also released into the

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extracellular milieu, potentially in an attempt to overcome the onslaught of toxic factors (Soontornniyomkij et al., 1998). Ultimately, with the progression of disease, the ensuing neuroinflammation disrupts neuronal function and leads to neuronal death. Importantly, *in vivo* studies have mirrored findings *in vitro*, showing an increase of both neurotoxic (*e.g.* TNF-α, MCP-1, IL-6) (Achim et al., 1996; Conant et al., 1998; Gisolf et al., 2000; Sippy et al., 1995) and neurotrophic (*e.g.* MIP-1α, FGF, BDNF) (Everall et al., 2001; Nuovo and Alfieri, 1996; Soontornniyomkij et al., 1998) factors in the cerebrospinal fluid (CSF) of HIV-infected patients. The precise mechanisms by which neuronal death occurs in response to this functionally diverse group of factors remain only partially defined; however, several mechanisms, including NMDA receptor stimulation (O'Donnell et al., 2006; Wang et al., 2007), calcium influx (Galicia et al., 2002), caspase and/or calpain activation (Wang et al., 2007), activation of p38 mitogen activated protein kinase (MAPK) and PI3K/Akt pathways (Ullrich et al., 2000), and/or aberrant cell cycle regulation (Jordan-Sciutto et al., 2002b) are suggested as determinants of neuronal viability in HAND. Interestingly, several neurodegenerative disorders, such as Alzheimer Disease (AD), Parkinson Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and HAND, display increased and/or altered expression of various cell cycle proteins in neurons, suggesting that cell cycle proteins may play important roles in determination of neuronal fate in disease (Jordan-Sciutto et al., 2001, 2003, 2002a, 2002b, 2000; Ranganathan and Bowser, 2003; Wang et al., 2010).

Retinoblastoma protein (pRb) is a key cell cycle regulator with the ability to target diverse signaling pathways and is therefore a major contributor to decisions about whether a cell divides, differentiates, senesces, or dies (Harbour and Dean, 2000a, 2000b). One of the essential events in cell cycle activation is hyperphosphorylation of pRb (ppRb) during the G1 phase of the cell cycle. In dividing cells, pRb prevents quiescent cells from entering the cell cycle through its repression of the transcriptional activity of cell cycle protein, E2F1 (Frolov and Dyson, 2004). The interaction between pRb and E2F1 and between pRb and other members of the E2F family of transcription factors is regulated by phosphorylation of pRb *via* CyclinD:cyclin dependent kinase (CDK)4, cyclinD:CDK6, or cyclin E:CDK2 complexes. pRb phosphorylation by one or more of CDKs abrogates pRb-mediated repression of the E2F family of transcription factors, leading to transactivation of a myriad of E2F target genes, including those necessary for DNA synthesis and S-phase progression, as well as those that induce cell death (DeGregori et al., 1997; Hallstrom and Nevins, 2003).

pRb is enriched in serines and threonines and is predicted to have over 30 potential phosphorylation sites, at least 12 of which are known to be phosphorylated *in vivo* (Connell-Crowley et al., 1997; Hamel et al., 1990; Kitagawa et al., 1996; Knudsen and Wang, 1996, 1997; Lees et al., 1991; Lin et al., 1991; Zarkowska and Mittnacht, 1997). While pRb phosphorylation at serine795 mainly regulates the disruption of the pRb:E2F1 complex, phosphorylation at serine807 and serine811 are involved in c-abl binding, and threonine821 and threonine826 have been shown to regulate binding to LxCxE-containing proteins (Knudsen and Wang, 1996). On the other hand, phosphorylation of pRb serine608 by Check point kinase (Chk) 1/2, in response to DNA damage, actually leads to formation of a complex between pRb and E2F1 (Inoue et al., 2007). Further, a recent report shows that pRb phosphorylation at threonine356/373 inhibits E2F1 transactivation domain binding to the pRb pocket domain. These vastly differing consequences of phosphorylation of pRb at different residues suggests that the effects of specific pRb phosphorylation events will have distinct impacts on cell fate.

One particular role for hyperphosphorylation of pRb that has come to light in recent years is its involvement in neurodegeneration. Several *in vitro* and *in vivo* studies have shown increased ppRb levels as evidence for aberrant cell cycle activation in various neurodegenerative processes. First, we have previously shown increased ppRb levels in neurons of patients afflicted with neurodegenerative disorders, including AD and PD (Burke et al., 2010; Jordan-Sciutto et al., 2003, 2002a), and in both HIV encephalitis (HIVE) (Jordan-Sciutto et al., 2002b), and a simian model of HIVE, SIVE (Jordan-Sciutto et al., 2000). *In vitro* studies have shown that forceful phosphorylation of pRb following cyclin D1 overexpression is sufficient to induce apoptosis of postmitotic neurons, further supporting a role for ppRb in disease (Freeman et al., 1994; Kranenburg et al., 1996). Additionally, other studies have demonstrated increased ppRb levels in post-mitotic neurons in response to trophic factor withdrawal, β-amyloid treatment, and oxidative stress (Giovanni et al., 1999; Liu and Greene, 2001; Padmanabhan et al., 1999), while inhibition of pRb phosphorylation by cyclin dependent kinases attenuated neuronal death induced by DNA damage and β-amyloid. However, we have also observed increased ppRb in murine neuronal cultures in response to the trophic factors, BDNF, NGF, and RANTES, with no apparent damage to the neurons (Strachan et al., 2005).

Given that several phosphorylation sites are known to have interactions with multiple proteins and considering that pRb function is dictated by its phosphorylation status, we reasoned that different stimuli may target discrete phosphorylation sites on pRb, leading to distinct outcomes in post-mitotic neurons, and might, therefore, explain the aforementioned contradictory observations regarding phosphorylation of pRb. In this study, we examined pRb phosphorylation patterns in primary mixed cortical cultures following treatment with trophic factors, Brain-derived neurotrophic factor (BDNF) and RANTES, or a neurotoxic factor, N-methyl D-aspartic acid (NMDA). Further, to determine whether any ppRb isotypes were specifically associated with HAND, we compared these phosphorylation patterns with those observed in cortical cultures in our *in vitro* model of HIV-induced neurodegeneration. Our results show that HIV-MDM leads to an early increase in ppRb serine795 *in vitro*, as assessed by western blotting (WB) and immunofluorescence (IFA); whereas pRb was phosphorylated at serine608 and serine 807/811 residues in RANTES- and BDNF-treated cultures. Both trophic factor-induced and HIV-MDM-induced pRb phosphorylation were dependent on global CDK activation; however, only BDNF-induced pRb phosphorylation was dependent on the specific CDK, CDK5, which has known roles in both neuronal growth and neuronal death. Further, overexpression of a mutant pRb nonphosphorylatable at serine795 attenuated neuronal damage in our model of HIV-induced neurotoxicity. Finally, consistent with our *in vitro* findings, we observed a significant increase in ppRb serine795 by immunofluorescence and immunoblotting in the mid-frontal cortices of autopsied brain tissue of HIV(+) individuals. Collectively, these data support our hypothesis that pRb is differentially phosphorylated in neurons depending on the stimulus and, more specifically, that it is preferentially phosphorylated on serine795 in association with HIV infection in the CNS, suggesting that the prevailing phospho-pRb isoform affects the survival of post-mitotic neurons. Our findings also suggest that pRb may be an important target for intervention in prevention of neuronal damage in HAND.

Results

pRb is differentially phosphorylated in response to the neurotoxic HIV-MDM, as compared with phosphorylation induced by trophic factors, RANTES and BDNF

Soluble neurotoxic factors, such as glutamate, quinolinic acid, and reactive oxygen species, as well as viral proteins released from HIV-infected monocyte-derived macrophages are proposed to be major determinants of neuronal death in HAND (Gendelman et al., 1994; Gonzalez-Scarano and Martin-Garcia, 2005b; Kaul et al., 2005). To recapitulate these conditions *in vitro*, we utilize a model in which we treat primary rat neuroglial cultures with supernatants (HIV-MDM) derived from monocyte-derived macrophages infected with a primary, macrophage tropic, HIV-1 isolate (Jago) collected from cell-

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