Lentivirus-associated MAPK/ERK2 phosphorylates EMD and regulates infectivity

Terence N. Bukong,1 William W. Hall1,2 and Jean-Marc Jacqué1,2,3

1University College Dublin, Centre for Research in Infectious Diseases, CRID Building, Belfield, Dublin 4, Ireland
2UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland
3Dundalk Institute of Technology, Department of Applied Sciences, School of Health and Science, Dublin Road, Dundalk, Co. Louth, Ireland

Infection of a cell by lentiviruses, such as human immunodeficiency virus type 1 or feline immunodeficiency virus, results in the formation of a reverse transcription complex, the pre-integration complex (PIC), where viral DNA is synthesized. In non-dividing cells, efficient nuclear translocation of the PIC requires the presence of the inner nuclear lamina protein emerin (EMD). Here, we demonstrate that EMD phosphorylation is induced early after infection in primary non-dividing cells. Furthermore, we demonstrate that EMD phosphorylation is dependent on virion-associated mitogen-activated protein kinase (MAPK). Specific inhibition of MAPK activity with kinase inhibitors markedly reduced EMD phosphorylation and resulted in decreased integration of the proviral DNA into chromatin. Similarly, when a MEK1 kinase-inactive mutant was expressed in virus-producer cells, virus-induced phosphorylation of EMD was impaired and viral integration reduced during the subsequent infection. Expression of constitutively active MEK1 kinase in producer cells did not result in modulation of EMD phosphorylation or viral integration during subsequent infection. These studies demonstrate that, in addition to phosphorylating components of the PICs at an early step of infection, virion-associated MAPK plays a role in facilitating cDNA integration after nuclear translocation through phosphorylation of target-cell EMD.

INTRODUCTION

Following receptor and co-receptor engagement on the target-cell by human immunodeficiency virus type 1 (HIV-1), viral nucleic acids are synthesized and translocated to the host cell’s nucleus as part of a ‘pre-integration complex’ (PIC). Lentiviruses, such as HIV-1, simian immunodeficiency virus and feline immunodeficiency virus (FIV), efficiently infect activated cycling T-cells in which the nuclear envelope is disassembled at mitosis. However, they have also evolved the ability to infect non-dividing cells in which the nuclear envelope remains intact at all times, such as T-cells in G1 of the cell cycle and beyond (Korin & Zack, 1998; Stevenson et al., 1990; Swingler et al., 2003; Zack et al., 1990). Thus, cytokines promoting progression of T-lymphocytes from G0 to G1 permit efficient lymphocyte infection (Unutmaz et al., 1999). HIV-1 appears to have evolved mechanisms to induce lymphocytes to cycle minimally thus rendering them susceptible to infection. For example, HIV-1-infected macrophages release soluble factors in a Nef-dependent manner that stimulate quiescent lymphocytes to enter G1 allowing them to become susceptible to infection (Swingler et al., 2003). In all of these non-dividing cells the nuclear membrane is an obstacle that has to be traversed in order to allow integration of the viral cDNA and a productive infection.

We recently reported that the inner nuclear envelope protein emerin (EMD) was required for virus infectivity in terminally differentiated macrophages (Jacque & Stevenson, 2006). While silencing other proteins of the nuclear lamina had little effect, silencing of EMD and another inner nuclear envelope protein, barrier to autointegration factor (BAF), resulted in the accumulation of 1- and 2-long terminal repeat (LTR) circles and fewer integration events as measured by PCR. Mutation within the LEM (LAP2–emerin–MAN1) domain of EMD, which binds BAF, resulted in the same effect as silencing. Subsequently, EMD was reported to be involved in
herpes simplex virus type 1 (HSV-1) infection (Leach et al., 2007; Morris et al., 2007). HSV-1 infection was shown to induce EMD phosphorylation and provoke redistribution of EMD within the nuclei of infected cells. Although the authors did not identify the kinase responsible, these studies demonstrated that the infection-induced phosphorylation of EMD was essential for viral DNA access to the nucleus (Leach et al., 2007; Morris et al., 2007).

In addition to proteins directing synthesis and integration of viral cDNA, HIV-1 PICs contain structural gag-MA (matrix) and -CA (capsid) proteins, the accessory protein Vpr (viral protein R) and at least one kinase identified as being ERK2/mitogen-activated protein kinase (MAPK) (Cartier et al., 1997; Jacque et al., 1998). Both gag-MA (Bukrinsky et al., 1993; von Schwedler et al., 1994) and Vpr (Heinzinger et al., 1994) facilitate the translocation of the PICs into the nuclei of non-dividing cells, allowing the establishment of HIV-1 infection. ERK2/MAPK was found to be specifically packaged into virions (Cartier et al., 1997; Jacque et al., 1998) and its activity was demonstrated to be required for gag-MA phosphorylation. Virus infectivity was substantially decreased in viruses in which ERK2/MAPK had been inhibited by specific kinase inhibitors. Similarly, expression of a dominant-negative mutant of MEK1 (MAPK-kinase) in producer cells significantly attenuated the extent of gag-MA phosphorylation and inhibited subsequent infection by HIV-1 (Jacque et al., 1998). Thus phosphorylation of at least one viral protein by ERK2/MAPK plays a vital role in facilitating viral infection.

In order to further elucidate the role of EMD in lentiviral infection, we examined the biochemical modifications elicited by HIV-1 and FIV infection in primary human macrophages and CD4+ T-cells. In the present study, as reported during HSV-1 infection, we demonstrate that target-cell nuclear protein EMD undergoes phosphorylation following lentiviral infection. Inhibition of virion-associated kinase with specific kinase inhibitors resulted in a sharp decrease in EMD phosphorylation and proviral integration. Likewise, when an inactive kinase mutant of MEK1 was expressed in the producer cell, preventing packaging of active ERK2/MAPK, the resulting virus progeny exhibited a decreased ability to induce EMD phosphorylation and integration of viral DNA. Thus, we identify the virion-associated ERK2/MAPK as the kinase responsible for EMD phosphorylation. Altogether, these data indicate that ERK2/MAPK plays an important role by promoting chromatin engagement of proviral DNA through EMD phosphorylation.

**RESULTS**

**Lentivirus infection induces EMD phosphorylation in primary non-dividing cells**

One of the main features that distinguish lentiviruses from other retroviruses is their ability to infect non-dividing cells, including cells of the monocyte/macrophage lineage and non-cycling T-cells, provided they are in or beyond the G1b stage of the cell cycle. We have previously demonstrated that EMD is required to enable access of viral DNA to chromatin in primary macrophages following HIV-1 infection. Here, we examined whether post-translational modifications of EMD were induced upon lentiviral infection. Primary monocytes were isolated and terminally differentiated into macrophages. Cells were infected with a vesicular stomatitis virus G-protein (VSVG)-pseudotyped HIV-1 (VSVG–HIV) or VSVG-pseudotyped FIV full-length molecular clone FIV-G8 (VSVG–FIV) for 1 h. Cells were harvested at the indicated times post-infection (p.i.) and lysed. Immunoprecipitations were performed with polyclonal antibodies against lamins A/C (LMNA/C), MAN antigen 1 (MAN1) or EMD (Fig. 1a, b) and proteins were analysed by Western blot with monoclonal antibodies. VSVG–HIV infection of macrophages did not elicit post-translational modification of LMNA/C or MAN1 (Fig. 1a). In contrast, while EMD was not modified in non-infected macrophages, mobility of a fraction of the protein appeared to up-shift as early as 4 h post-infection in cells infected with either VSVG–HIV or VSVG–FIV viruses (Fig. 1b). When immunoprecipitates were pre-incubated for 30 min in the presence of recombinant protein phosphatase-1 (PP1) the shifted species were removed, demonstrating that the mobility shift was due to phosphorylation. Altogether these results indicate that upon lentivirus infection of macrophages EMD phosphorylation is induced within a time frame consistent with nuclear translocation of the PICs following reverse transcription.

Another major non-dividing target-cell type for lentivirus the CD4+ T-cell, mainly memory T-cells in the G1-stage of the cell cycle and beyond. Therefore, we examined whether EMD was similarly modified in infected G1-arrested T-cells. Purified CD4+ T-cells were activated overnight on CD3-coated plates with a CD28 antibody. Cells were then arrested in G1 by incubation with a CD26 antibody (Mattern et al., 1993; Ohnuma et al., 2002). G1-arrested T-cells were infected with VSVG–HIV or VSVG–FIV at the indicated times and harvested (Fig. 1c). EMD was immunoprecipitated from cell lysates and subjected to immunoblotting. EMD appeared to be modified as early as 4 h p.i. for VSVG–HIV and 8 h p.i. for VSVG–FIV. PP1 treatment removed the mobility shift indicating that EMD had been phosphorylated. Heat-inactivated virus (hi) had no effect on EMD phosphorylation at 8 h indicating that virus entry was necessary (Fig. 1c).

In addition, when different multiplicities of infection were used to infect cells, EMD phosphorylation appeared to be reduced when five times less virus was used indicating that efficient infection was required to induce this effect (Fig. 1d).

**Modulating ERK2/MAPK activity inhibits virus-induced EMD phosphorylation and proviral integration in primary macrophages**

We next attempted to identify the kinase responsible for EMD phosphorylation. It has previously been reported that
ERK2/MAPK was specifically incorporated into virions and was necessary for virus infectivity (Cartier et al., 1997; Jacque et al., 1998). We therefore tested the hypothesis that MAPK could be responsible for EMD phosphorylation. FR180204 is an ERK1/2-specific inhibitor that directly prevents the kinase from phosphorylating substrates (Ohori et al., 2005). Primary human macrophages were treated with FR180204 or staurosporine, a broad-spectrum protein kinase inhibitor that is inefficient at blocking MAPK activity (Couldwell et al., 1994; Nishimura & Simpson, 1994), for 1 h prior to VSVG–HIV infection (Fig. 2a). FR180204 dramatically inhibited VSVG–HIV infection-induced EMD phosphorylation at 0.51 μM (IC50) and above. In contrast, staurosporine had no effect upon EMD phosphorylation at 7 nM [its IC50 for protein kinase A (PKA)] and little effect up to 700 nM. A decrease in infection-induced EMD phosphorylation was also observed following VSVG–FIV infection of macrophages in the presence of FR180204 but not in the presence of staurosporine (Fig. 2b).

In the MAPK cascade MEK1/2 are the only known direct activators of ERK1/2 and are believed to phosphorylate only these two substrates (Robinson et al., 1996). In order to establish whether kinase activity could be attributed to endogenous ERK2/MAPK or the PIC-associated kinase, we took a similar approach to that previously reported (Jacque et al., 1998).
et al., 1998). VSVG–HIV viruses were produced in the presence of vectors, along with the viral components, expressing no MEK1 (MEK-WT), a constitutively active MEK1 (MEK-SA) or a kinase-inactive MEK1 mutant (MEK-DN) (Mansour et al., 1994) (Fig. 2c). The role of gag-MA phosphorylation in viral infectivity remains controversial. In order to bypass the possible need for gag-MA phosphorylation for efficient uncoating, we designed an in vitro approach to specifically evaluate nuclear transfer of PICs and integration of viral cDNA. The viral envelope was disrupted to isolate virus cores. Cell membranes of primary macrophages were also disrupted to allow direct interaction between viral cores and cell nuclei. Proper integration of viral cDNA was not impaired under these conditions, as shown by the efficiency of MEK-WT core infection (Fig. 2c, WT). When viral cores produced in the presence of
MEK-WT or MEK-SA were added to macrophage nuclei. EMD phosphorylation was unaffected. In contrast, a sharp decrease in EMD phosphorylation was observed when MEK-DN was used to generate viruses (Fig. 2c). PP1 treatment removed the additional species confirming EMD phosphorylation (Fig. 2c). Azidothymidine (AZT) pre-incubation of the cells also inhibited EMD phosphorylation indicating that full-length cDNA synthesis was required for proper nuclear entry and interaction with the nuclear lamina (Fig. 2c).

To date, incorporation of ERK2/MAPK into FIV particles has not been reported. We therefore wished to verify that the kinase was also associated with FIV virions. Virions were isolated from HIV-1- or FIV-G8-transfected 293T cells and viruses were treated with subtilisin to remove cell membrane vesicle contamination as previously described (Ott, 2009). Pellets were lysed and proteins separated by SDS-PAGE and transferred to nitrocellulose. Membranes were prepared and probed with p24 antibodies to FIV and HIV-1 or an ERK2/MAPK antibody, revealing that the kinase was present in both HIV-1 and FIV particles (Fig. 2d).

**Modulating ERK2/MAPK activity inhibits virus-induced EMD phosphorylation and proviral integration in primary G1-arrested CD4\(^+\) T-cells**

We compared the ability of lentiviruses to induce EMD phosphorylation in CD4\(^+\) T-cells at different stages of the cell cycle (Fig. 3). Cells in G0 are refractory to infection. No post-translational modifications of EMD were observed in cells in G0 8 h p.i. In contrast, G1-arrested T-cells exhibited a strong EMD mobility shift 8 h p.i. similar to the one observed in macrophage infections. In cycling T-cells, containing cells at various stages of the cell cycle, the EMD shift was significantly reduced compared with the G1-arrested population (Fig. 3a).

The band shift was caused by EMD phosphorylation as confirmed by PP1 treatment (Fig. 3b). We then assessed the effect of FR180204 and staurosporine on the phosphorylation status of EMD in HIV-infected or non-infected G1-arrested CD4\(^+\) T-cells (Fig. 3b). FR180204 had little effect on EMD phosphorylation at 0.051 μM but strongly inhibited EMD phosphorylation at 0.51 μM. In contrast, staurosporine had little effect on the phosphorylation status of EMD in these T-cells. Although the T-cells used in this study were positively selected, it is unlikely that CD4 cross-linking had any effect on EMD phosphorylation since there was no difference in phosphorylation of EMD in G0 cells whether they were infected or not (Fig. 3a). In addition, no EMD phosphorylation was observed in uninfected cycling or G1-arrested T-cells.

G1-arrested and cycling T-cells were analysed with the carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution method in order to confirm that G1-arrested cells were not dividing (Fig. 3c).

Altogether these results suggest that, in the non-dividing populations of macrophages and G1-arrested T-cells, EMD is phosphorylated by the virion-associated ERK2/MAPK shortly after lentivirus infection.
Inhibition of ERK2/MAPK-induced EMD phosphorylation decreases lentivirus infectivity in primary non-dividing cells

We examined the role of EMD phosphorylation in regulating virus infectivity in primary non-dividing cells. VSVG–HIV–GFP and VSVG–FIV–GFP were produced in 293T cells and incubated with target primary macrophages in the presence or absence of FR180204 or staurosporine. Infections proceeded for 48 h before cells were analysed by flow cytometry for GFP expression. While non-treated (WT) or staurosporine-treated (Stau, 7 nM) cells were efficiently infected, cells treated with FR180204 (FR) exhibited a strong resistance to both VSVG–HIV–GFP and VSVG–FIV–GFP infections as assessed by GFP expression. Pretreatment with AZT, blocking reverse transcription and therefore integration, also abolished infection (Fig. 4a).

To evaluate the role of virus-associated ERK2/MAPK in regulating infectivity, GFP-expressing viruses were also produced in the presence of MEK-SA or MEK-DN. Infectivity was unaltered in viruses produced in the presence of the endogenous active ERK2/MAPK or the constitutively active MEK-SA mutant (Fig. 4b). In contrast, when the inactive mutant MEK-DN was used to produce viruses containing inactive ERK2/MAPK, virus infectivity was strongly inhibited to levels that were comparable to blocking viral cDNA synthesis with AZT.

We also examined the effects of kinase inhibitors or MEK mutants on viral infectivity in primary G1-arrested CD4+ T-cells. Similar to the observations in primary macrophages, FR180204 and MEK-DN expression strongly inhibited virus infectivity while staurosporine or MEK-SA expression had no effect on proviral DNA integration and virus expression in VSVG–HIV–GFP or VSVG–FIV–GFP infections (Fig. 4c, d).

Altogether, our data indicate that EMD phosphorylation by ERK2/MAPK is required for efficient infection by allowing access of viral nucleic acids to the nuclear compartment.

Inhibition of virus-associated ERK2/MAPK activity decreases proviral integration

Proviral integration into host chromatin is a prerequisite for efficient viral expression. We therefore assessed the effect of ERK2/MAPK inhibitors or expression of MEK mutants on viral cDNA integration into the host chromatin. Macrophages or G1-arrested CD4+ T-cells were infected with VSVG–HIV or VSVG–FIV. Total DNA was extracted from cells and subjected to real-time PCR analysis for integrated proviral DNA, 2-LTR circle species or total viral DNA (Total LTR). Similarly to our infectivity data, FR180204 strongly inhibited viral DNA integration to levels comparable to AZT pretreatment while staurosporine had little effect. FR180204 treatment also resulted in a sharp increase in 2-LTR circle species formation similar to that observed in our previous study in EMD-silenced cells (Jacque & Stevenson, 2006). Neither of the kinase inhibitors affected reverse transcription since total cDNA (Total LTR) was unchanged while AZT efficiently blocked cDNA synthesis. Agarose gel analysis of semiquantitative PCR confirmed those results (Fig. 5a, b).

We then infected macrophages with viruses produced in the presence or absence of the MEK1 mutants. Viruses produced in the presence of MEK-DN displayed a strong integration defect and as a consequence produced more 2-LTR circles, which are results similar to those observed when ERK2/MAPK was inhibited by FR180204. The presence of MEK mutants had no apparent effect on the ability of viruses to synthesize cDNA since total LTR species were comparable. In contrast, AZT strongly inhibited cDNA synthesis. Semiquantitative analysis by gel electrophoresis showed similar results to those obtained by real-time PCR analysis (Fig. 5c).

These results suggest that, in the non-dividing populations of macrophages and G1-arrested T-cells, the phosphorylation of EMD by ERK2/MAPK appears to be required for efficient integration of the proviral DNA into host chromatin.

**Fig. 4.** Modulation of ERK2/MAPK activity affects virus infectivity in primary macrophages and G1-arrested CD4+ T-cells. (a, c) Primary human macrophages (a) or G1-arrested T-cells (c) pretreated or not with FR180204, staurosporine or AZT were infected or not (NI) with VSVG–HIV–GFP or VSVG–FIV–GFP viruses for 48 h and analysed by flow cytometry for GFP expression. (b, d) Primary human macrophages (b) or G1-arrested T-cells (d) were infected or not (NI) with VSVG–HIV–GFP produced in the absence (WT) or presence of the constitutively active (SA) or dominant-negative (DN) MEK1 mutants for 48 h and analysed by flow cytometry for GFP expression.
Fig. 5. Modulation of ERK2/MAPK activity affects proviral integration in primary lentiviral targets. (a, b) Primary human macrophages (a) and G1-arrested T-cells (b) were pretreated or not with FR180204, staurosporine or AZT and infected or not (NI) with VSVG–HIV for 48 h. Graphs: real-time PCR analysis was performed for integrated DNA, 2-LTR circle species and total proviral DNA (Total LTR). Gels: semiquantitative PCR analysis of integrated compared with total proviral species (Total LTR). (c) Primary human macrophages were infected or not (NI) with VSVG–HIV produced in the absence (WT) or presence of the constitutively active (SA) or dominant negative (DN) MEK1 mutants for 48 h. Graphs: genomic DNA was extracted and real-time PCR analysis was performed as above. Gels: semiquantitative PCR analysis of integrated compared with total proviral species (Total LTR).
Recombinant activated ERK2/MAPK phosphorylates EMD in vitro

Finally, we examined whether activated recombinant ERK2/MAPK was able to phosphorylate EMD in vitro. EMD was immunoprecipitated from infected or non-infected primary macrophages. Immunoprecipitates from non-infected cells were incubated in the presence or absence of active ERK2/MAPK and with or without PP1 or FR180204. Immunoprecipitates from infected cells were treated, or not, with PP1. Active recombinant ERK2/MAPK phosphorylated EMD, an effect inhibited by FR180204 at 0.51 or 5.1 μM and removed by PP1 (Fig. 6).

These data strongly suggest that ERK2/MAPK is the main kinase responsible for lentivirus infection-induced phosphorylation of EMD.

DISCUSSION

The results outlined in this study show that EMD phosphorylation is a required event in the pathway leading to proviral cDNA integration following infection of non-dividing target-cells by lentiviruses. In addition, our study shows that EMD phosphorylation is induced by the virus-associated ERK2/MAPK at a point prior to proviral integration. Cells treated with the specific ERK2/MAPK inhibitor FR180204 exhibited reduced EMD phosphorylation and this correlated strongly with inhibition of viral integration. Similarly, in cells infected with viruses containing inactive ERK2/MAPK, i.e. viruses produced in the presence of MEK-DN mutants, both EMD phosphorylation and proviral integration were inhibited. The data we present here strongly correlate with our previous, separate, findings that ERK2/MAPK and EMD play an important role in determining HIV-1 infectivity (Jacque et al., 1998; Jacque & Stevenson, 2006). In the present study we demonstrate that these two processes are linked. The data consistently show that inhibition or elimination of virus-associated ERK2/MAPK activity reduces EMD phosphorylation, resulting in decreased proviral integration. The data have been confirmed in two primary human non-dividing cell targets, macrophages and G1-arrested T-cells, infected by two distinct lentiviruses, HIV-1 and FIV. Therefore, we postulate that lentiviruses have evolved a general mechanism to assure efficient infection of non-dividing cells in their hosts, using EMD to ensure proper access to the nuclear compartment in cell types in which the nuclear membrane would otherwise present a major barrier.

We reported recently that the nuclear lamina proteins EMD and BAF are necessary for HIV-1 infection of non-dividing cells (Jacque & Stevenson, 2006). Silencing of EMD resulted in the accumulation of 1- and 2-LTR circles and a decreased integration of the proviral DNA into the chromatin of the host cells. We have since observed that EMD is also required for FIV infection (J.-M. Jacqué, unpublished data). Other authors also report that viruses, such as HSV-1, gain access to the nucleus through their ability to phosphorylate EMD. However, the kinase responsible for this modification was not identified (Leach et al., 2007; Morris et al., 2007). In the latter study HSV-1 infection-induced EMD phosphorylation caused EMD delocalization, an observation that remains to be assessed in lentiviral infections. Other viruses are also reported to achieve access to the nuclear compartment by phosphorylation of nuclear lamina proteins. It has been shown that B1 kinase encoded by vaccinia virus induces the phosphorylation of BAF, rather than EMD, allowing proper replication of the virus (Nichols et al., 2006; Wiebe & Traktman, 2007). Other viruses such as HSV-1 may also encode their own kinase able to phosphorylate EMD or other substrates required at different stages of their life cycle. Interestingly, small viruses such as lentiviruses appear to have evolved to interact with and encapsidate at least one cellular kinase instead of encoding their own. It appears that viruses infecting non-dividing cells have evolved a general mechanism involving phosphorylation of nuclear proteins with the common outcome of allowing viral DNA access to the nuclear compartment.

This study was performed using VSVG-pseudotyped viruses. There is accumulating evidence that such viruses do not enter cells in the same way as wild-type enveloped viruses and that the infection outcome and replicating properties of those viruses are different (Agosto et al., 2009; Yu et al., 2009). However, in our previous study (Jacque & Stevenson, 2006), we addressed this issue, comparing the replication and integration of VSVG- and wild-type enveloped HIV-1 viruses. EMD, BAF and lamina-associated protein 2α (LAP2α) were silenced in separate experiments. Silencing of EMD and BAF inhibited replication and integration of both virus models. In contrast, when LAP2α was silenced these processes were only inhibited in the wild-type virus. Although some differences in replication properties were observed these did not affect the EMD requirement. Thus, we believe that the use of pseudotyped viruses in this study is unlikely to have influenced the necessity for EMD.
phosphorylation for proper chromatin engagement in primary non-dividing cells.

The importance of the role of EMD in HIV infectivity has been controversial. One recent study reported that EMD was not required for infection of HeLa cells and its silencing had little effect in macrophage infection (Shun et al., 2007). Most of that study focused on HeLa cells, which are rapidly dividing and in which disassembly of nuclear membranes during mitosis enables viral DNA access to chromatin, thus avoiding any need for modification of EMD. However, the authors performed one experiment on monocytes and found a modest effect of EMD silencing after one round of transfection. Our results showed a much stronger effect after two rounds of transfection in macrophage colony-stimulating factor (M-CSF) terminally differentiated macrophages. We have proposed that the use of EMD may be restricted to terminally differentiated non-dividing cells of the macrophage lineage and non-cycling T-cells in G1. The data presented herein, linking phosphorylation of EMD with viral integration, strongly support the role of EMD in allowing efficient infection of these non-dividing cells and strongly support our previous data that EMD is required for infectivity. A second study also questioned the role of EMD in mediating HIV-1 infectivity. The authors showed that transgenic EMD-knockdown mouse fibroblasts could be infected ex vivo by HIV (Mulky et al., 2008). However, upon cell division, EMD-deficient cells exhibit abnormal reassembly of the nuclear membrane, resulting in chromatin protrusions across the nuclear membrane (Lammerding et al., 2005). Such protrusions expose the chromatin to the cytoplasm; therefore the nuclear membrane cannot present a barrier to proviral DNA integration into the chromatin of the host cells. In addition, the efficient infection of mouse cells by HIV-1 is subject to many parameters that include expression of human cyclin T1 and other human proteins in a xenogenic environment that could influence the infection outcome. For example, as previously reported, BAF is required for efficient infection. In knockdown transgenic mice, BAF could play a major role in allowing integration since cells must reorganize their nuclear membrane structure in the absence of EMD (Bengtsson & Wilson, 2004; Chen & Engelman, 1998; Jacque & Stevenson, 2006). The findings we report here strongly support our previous data that EMD is required for infectivity.

In this study we identify ERK2/MAPK as the kinase responsible for EMD phosphorylation following viral entry. Moreover, our data point at the involvement of virion-associated ERK2/MAPK rather than the endogenous kinase in target-cells. ERK2/MAPK is a proline-directed kinase (Davis, 1993). EMD contains at least three putative (albeit weak) ERK2/MAPK consensus sequences and recombinant ERK2/MAPK was able to phosphorylate EMD in vitro in a manner inhibitable by FR180204. Interpretation of data using kinase inhibitors can be challenging given the multiplicity of substrates within the cell. For example, we used staurosporine as a non-specific kinase inhibitor because it inhibits a broad range of cellular kinases including PKA (IC50 7 nM), calmodulin-dependent protein kinase (CaM) (IC50 20 nM), myosin light chain kinase (IC50 1.3 nM), protein kinase C (PKC) (IC50 700 pM) and protein kinase G (IC50 8.5 nM). In contrast, it has been reported to have little or no effect on HSV-1-induced EMD phosphorylation (Morris et al., 2007). In VSVG–HIV infections, 7 nM staurosporine had minimal effect on EMD phosphorylation; however, a twofold decrease in proviral integration into host chromatin was detected. This reduction could be explained by a pleiotropic effect due to the broad range inhibitory activity of staurosporine. In this experiment, we used the inhibitor at 7 nM, 10 × IC50 for PKC. PKC has also been reported to modulate virus infectivity in a complex way (Warrior et al., 2006). Therefore this observation may be due to an effect of PKC inhibition upon a post-nuclear entry, pre-integration event that would have to be further investigated. FR180204 may also exhibit pleiotropic effects in the target-cells due to the presence of many different substrates for ERK2/MAPK. Our use of MEK1 mutants in the producer cells allows us to control whether the virion-associated kinase is active. Inactive kinase elicits the same response as treatment with FR180204. Our previous work with wild-type and mutant MEK1 strongly suggests that ERK2/MAPK has to be associated with the PICs (Jacque et al., 1998). As a result, we anticipate that virion-associated kinase activity is restricted to substrates targeted by the PICs rather than exhibiting a non-specific effect on many cellular substrates. Therefore, we suggest that the effect observed with FR180204 is due to its modulation of the PIC-associated kinase rather than a pleiotropic effect. However, it has previously been shown that virion-associated ERK2/MAPK induces phosphorylation of the viral protein gag-MA thus regulating infectivity (Cartier et al., 1997; Jacque et al., 1998). We have unveiled here another role for virion-associated ERK2/MAPK in its ability to phosphorylate at least one cellular protein, EMD. There is a possibility that other cellular substrates may also be specifically targeted by the virion-associated kinase. However, we show a constant correlation between the loss of kinase activity and EMD phosphorylation affecting viral DNA integration after reverse transcription, pointing at a key role for EMD in allowing successful viral infection in these cells. This study also reports for the first time that FIV similarly encapsidates ERK2/MAPK. The exact mechanism by which ERK2/MAPK interacts with HIV or FIV remains to be elucidated. The fact that two members of the lentivirus family specifically encapsidate ERK2/MAPK raises the possibility that this enzyme plays a central role in the regulation of the lentiviral life cycle. It could be argued that specifically encapsidating a cellular kinase that could be used at different stages of the viral life cycle would be strategically beneficial.

Taken together, this set of data suggests that the activity of virion-associated ERK2/MAPK is necessary for the establishment of a functional reverse transcription complex within the target-cell at the level of nuclear localization of the PICs in order to allow proper integration into the host cell chromatin. These observations point to an essential
interaction between virion-associated ERK2/MAPK and host EMD in the virus life cycle. Thus, our findings also provide a rationale for strategies which block HIV-1 infection prior to proviral integration, by antagonizing the interaction of the virus-associated MAPK with EMD.

**METHODS**

**Cells.** Buffy coats were obtained from the Irish Blood Transfusion Services (IBTS) and peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Paque density gradient (GE Healthcare). Monocytes were isolated by plastic adherence. Monocyte-derived macrophages (MDMs) were differentiated for 7 days with 10 ng M-CSF ml⁻¹ (R&D systems) as described previously (Jacque & Stevenson, 2006; Swingler et al., 1999, 2003). Cycling T-cells were prepared by incubating PBMCs with 5 μg phytohaemagglutinin ml⁻¹ (Sigma) for 72 h and maintained in complete RPMI 1640 medium at 37°C for overnight and arrested in G1 with 1 μg mitomycin) and 1 μg anti-CD26 ml⁻¹. Lymphocyte activation was confirmed by the CFSE dye dilution method (Molecular Probes) (Lyons & Parish, 1994). CD4⁺ T-cells were positively selected with anti-CD4 immunoaffinity columns (Miltenyi). For activation, plates were coated with CD3 monoclonal antibodies at 1 μg ml⁻¹. Cells were seeded (10⁶ per well) in RPMI medium and 1 μg anti-CD28 ml⁻¹ was added. Cells were cultured overnight and arrested in G1 with 1 μg anti-CD26 ml⁻¹ (Mattern et al., 1993; Ohnuma et al., 2002) or left cycling. Cycling status of the cells was assessed by the CFSE dye dilution method as above.

**Viruses production and infection.** 293T cells were transfected with 20 μl Lipofectamine 2000 (Invitrogen). VSVG–HIV viruses were generated using 1 μg p8.91 HIV-lag-pol, 1.5 μg pSIN CSPW (puromycin) and 1 μg pMDG–VSVG expression vector. ERK2/MAPK mutant-containing virions were generated by adding 1 μg MEK-DN or MEK-SA expression plasmids to the transfection mix. VSVG–FIV virions were prepared by cotransfecting 10 μg FIV-G8 molecular clone and 7 μg pMDG–VSVG. Non-pseudotyped viruses for Western blotting were produced using 1 μg p8.91 HIV-lag-pol and 1.5 μg pSIN CSPW (puromycin) or 10 μg FIV-G8 molecular clone. All viruses were collected after 48 h.

**ERK2/MAPK presence in viruses.** Culture supernatants containing HIV-1 p24 immunocytansfected, FIV–G8–transfected or untransfected 293 T-cells were filtered on 0.2 μm membranes and precleared at 600 g. Supernatants were treated or not with 1 μg subtilisin ml⁻¹ as described (Sova et al., 2001). Viruses were pelleted at 18 000 g for 80 min at 4°C. Pellets were resuspended in 20 μl total protein lysis buffer containing protease inhibitors (Sigma). Samples were denatured at 100°C with 20 μl Laemmli sample buffer (Sigma). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare) and probed with mouse anti-human ERK2 antibody (C14; Santa Cruz).

**Nuclear protein extractions.** Cells were lysed in 1 ml buffer N (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM DDT) containing 10 μg protease inhibitor cocktail ml⁻¹ (PI) (Sigma). Nuclei were pelleted at 3000 g and resuspended in lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate) with PI and centrifuged at 13 000 g. 20 ng of nuclear proteins were subjected to SDS-PAGE (10% polyacrylamide gel) and transferred to membranes for immunoblotting.

**Immunoprecipitations.** Cells were infected with viruses in the presence or absence of kinase inhibitors (Merck). Macrophages were lysed at the indicated times for 1 h in 1 ml ice-cold lysis buffer as above. Soluble material was preclared with 20 μl Protein A/G–Agarose beads(Santa Cruz Biotechnology). After overnight incubation with 3 μg anti-EMD antibody (Santa Cruz Biotechnology) 20 μl Protein A/G–Agarose was added for 2 h. Beads were washed in PBS (pH 7.4) and subjected to SDS-PAGE (10% polyacrylamide gel) and transferred. For PP1 (Roche) treatment, immunoprecipitates were incubated with the enzyme for 30 min prior to SDS-PAGE.

**Immunoblotting.** Membranes were processed as described previously (Jacque et al., 1998; Jacque & Stevenson, 2006) and revealed by enhanced chemiluminescence (Pierce).

**PCR analysis of total and integrated HIV-1 DNA.** Total genomic DNA was extracted with a DNaseasy kit (Qiagen). PCRs were performed as previously described on normalized amounts of DNA (Jacque & Stevenson, 2006). Genomic DNA of HeLa cells with an LTR–TAT–GFP construct stably integrated in the genome (one copy per cell) was used as a standard. PCR was performed with SYBR Green kits (Qiagen) according to the manufacturer’s specification or using KOD Hot Start DNA Polymerase (Novagen). For integrated DNA purification a nested PCR technique was used. First-round PCR used primers SB704 (Alu) (5’–TGCTGGGATTACAAGCGTG–3’) and Rc (LTR) (5’–TAGACGATCAGCGCTGGGA–3’). PCR was performed in the presence of 500 ng genomic DNA, 0.2 mM dNTPs, 0.3 μM primers and KOD DNA Polymerase kit reagents in 50 μl reactions. Second-round PCR was performed on 1% of first-round PCR using the SYBR green kit with primers U3–47: (5’–GTTGAGATCCACACACACAAAGG–3’) and U5–500R: (5’–GGAGTGTGTCTCTAGT–3’) in a 50 μl reaction. Total LTR amplification was performed on 500 ng genomic DNA with primers U3–47: (5’–GTTGAGATCCACACACACACAGG–3’) and U5–500R: (5’–GGAGTGTGTCTCTAGT–3’) in a 50 μl reaction using SYBR green. 2-LTR amplifications were carried out with primers 2LTR-F: (5’–GTAAGTTTGCGCCCTGT–3’) and 2LTR-R: (5’–CTCCTTCTTGAGTGTAATGC–3’).

**In vitro kinase assays.** EMD immunoprecipitates were incubated with active recombinant ERK2/MAPK (Millipore) in the presence or absence of PP1 or FR180204 for 1 h prior to denaturation in Laemmli buffer and SDS-PAGE analysis.

**ACKNOWLEDGEMENTS**

We thank B. J. Willett (Institute of Comparative Medicine, University of Glasgow, Glasgow, UK) for the FIV-G8 molecular clone, M. Naghavi (University College Dublin, Centre for Research in Infectious Diseases, Dublin 4, Ireland) for the HIV-p24immunocytansfected, vesicular stomatitis virus clones and N. Ahn (Department of Chemistry and Biochemistry, University of Colorado at Boulder, CO 80309, USA) for MEK expression vectors. We thank S. Emiliani for critical reading of the manuscript and helpful discussions. 293T clone 17 cells were from the ATCC. This work was supported by a start-up grant from University College Dublin to J.-M.J. The authors declare no competing commercial interests.

**REFERENCES**


Regulation of HIV infectivity by ERK2/MAPK


