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Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase

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Infection of a cell by human immunodeficiency virus type 1 (HIV-1) results in the formation of a reverse transcription complex in which viral nucleic acids are synthesized. Efficient disengagement of the reverse transcription complex from the cell membrane and subsequent nuclear translocation require phosphorylation of reverse transcription complex components by a virion-associated kinase. In this study, we identify the virion-associated kinase as mitogen-activated protein kinase (ERK/MAPK). Upon density gradient fractionation, MAPK, but not its activating kinase MEK, co-sedimented with viral particles. Expression of a constitutively active, but not kinase-inactive, MEK1 in virus producer cells was able to activate virion-associated MAPK *in trans*. Stimulation of virion-associated MAPK activity *in trans* by the mitogen phorbol myristate acetate (PMA) increased viral infectivity. Conversely, suppression of virion-associated MAPK by specific inhibitors of the MAPK cascade markedly impaired viral infectivity. These studies demonstrate regulation of an early step in HIV-1 infection by the host cell MAPK signal transduction pathway.

Keywords: HIV infectivity/MAP kinase

Introduction

Following CD4/co-receptor engagement on the target cell by HIV-1, viral nucleic acids are synthesized and translocated to the host cell nucleus within the context of a nucleoprotein reverse transcription complex (reviewed in Stevenson, 1996). Synthesis and translocation of viral nucleic acids from the point of virus entry to the nucleus are dependent upon activation of the host cell. As a consequence, quiescent T cells do not support efficient HIV-1 infection (Stevenson *et al.*, 1990; Zack *et al.*, 1990; Bukrinsky *et al.*, 1991; Gao *et al.*, 1993; Spina *et al.*, 1995; Chun *et al.*, 1997; Sun *et al.*, 1997). In addition to proteins which direct synthesis and integration of viral nucleic acids, HIV-1 reverse transcription complexes contain structural gag matrix (MA) protein (Bukrinsky *et al.*, 1993a; Karageorgos *et al.*, 1993; Heinzinger *et al.*, 1994; Farnet and Bushman, 1997) and the accessory protein Vpr (Heinzinger *et al.*, 1994; Fletcher *et al.*, 1996), both of which are derived from the virion. Vpr is packaged within HIV-1 virions in stoichiometrically equivalent amounts relative to gag capsid (CA) through its interaction with gag p6 (Lu *et al.*, 1993; Paxton *et al.*, 1993). The association of primate lentiviral gag MA and Vpr proteins with viral reverse transcription complexes suggests that these proteins function at an early stage in the virus life cycle, i.e. at a point preceding *de novo* synthesis of viral proteins. Previous studies have demonstrated that gag MA (Bukrinsky *et al.*, 1993b; von Schwedler *et al.*, 1994) and Vpr proteins (Heinzinger *et al.*, 1994) promote the nuclear translocation of viral reverse transcription complexes in non-dividing cells, a process which is important for the establishment of HIV infection in terminally differentiated macrophages (reviewed in Stevenson, 1996).

The association of gag MA protein with the HIV-1 reverse transcription complex as it translocates to the host cell nucleus is paradoxical. Gag MA is myristoylated and this modification is required for efficient membrane localization of gag polyproteins prior to virus assembly (Gheysen *et al.*, 1989; Göttlinger *et al.*, 1989; Bryant and Ratner, 1990). Thus, during virus replication, the gag MA protein carries out opposing targeting functions in that membrane interaction is required for viral assembly while association with reverse transcription complexes is important for viral infectivity. Recent studies have indicated that the opposing targeting functions of gag MA are regulated by phosphorylation (Gallay *et al.*, 1995; Bukrinskaya *et al.*, 1996). It was further demonstrated that phosphorylation of gag MA was effected by a virion-associated serine/threonine kinase and that this phosphorylation promoted membrane dissociation of the reverse transcription complex (part of the uncoating process), a necessary prerequisite for subsequent nuclear translocation (Bukrinskaya *et al.*, 1996). However, the identity of the virion-associated kinase which was acting at this early step in HIV-1 replication and its actual role in the HIV-1 entry process were not known. Studies presented here indicate that ERK/MAPK (Boulton *et al.*, 1991; Robinson and Cobb, 1997) associates with highly purified virions of HIV-1, that this kinase promotes phosphorylation of gag MA proteins in the virion, and that modulation of virion-associated ERK/MAPK activity influences HIV-1 infectivity at an early step in the virus life cycle.

Results

ERK/MAPK associates with HIV-1 virions

Gradient-fractionated virion preparations were probed with antibodies specific to components of the ERK/MAPK (Boulton *et al.*, 1991), JNK/SAPK (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994) and p38/HOG-1 (Han *et al.*, 1994) kinase cascades in order to determine whether any com-

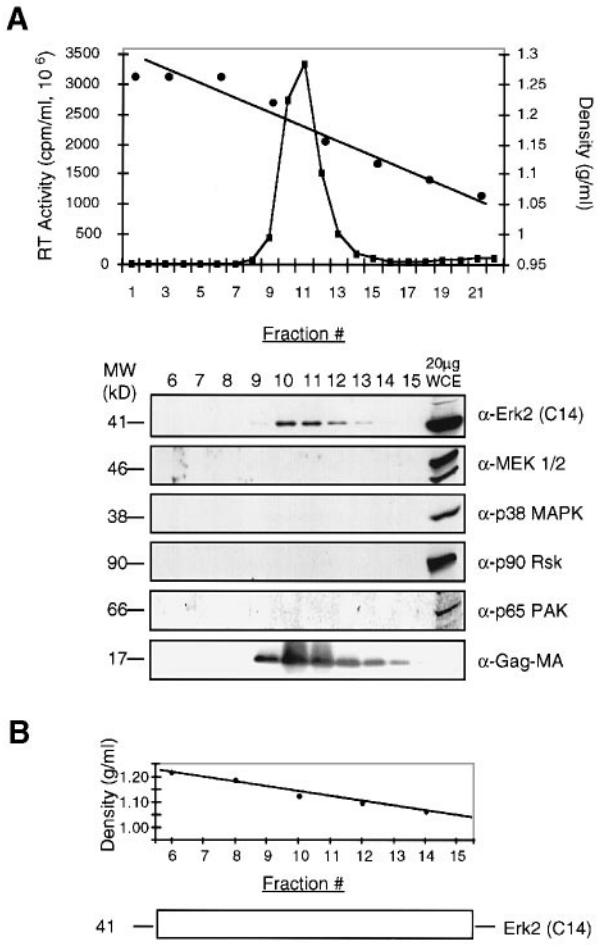

Fig. 1. Association of ERK/MAPK with HIV-1 virions. (**A**) Virus particles were obtained from productively infected MT-4 cells, purified and banded on sucrose density gradients as outlined in Materials and methods. Gradients were collected in 1 ml aliquots, 10 µl were used directly for determination of HIV-1 associated RT activity. Virus particles in the remaining fraction were pelleted and analyzed by Western blot analysis with the indicated antibodies. Twenty µg of whole-cell extract (WCE) was examined in parallel. Comparison of the levels of ERK/MAPK proteins with RT activity and with HIV-1 gag MA proteins in each gradient fraction indicates co-localization of ERK/MAPK with virus particles. (**B**) Culture supernatants of uninfected MT-4 cells were processed exactly as for infected culture supernatants, resolved on sucrose density gradients and examined for the presence of ERK/MAPK by Western blotting with an α ERK 2 (C14) antibody. All Western blot images are within the linear range of the ECL assay.

ponents of these signal transduction cascades co-localized with virion proteins following density gradient fractionation of virus particles. HIV-1 virions were obtained from productively infected T-cell lines and from PHA-activated PBMCs prior to the onset of viral cytopathic effects. Viral supernatants were clarified (1500 *g* for 10 min followed by filtration, 0.22 µm), virions were pelleted and then resolved on a 15–60% (w/v) continuous sucrose density gradient (200 000 *g* for 6 h). Under these conditions, virions [detected by reverse transcriptase (RT) activity] sediment at 1.17–1.18 g/ml. Individual gradient fractions were examined by Western blotting with antibodies to the structural gag MA protein and to the indicated kinases. ERK/MAPK co-localized with virion-associated RT activity and with structural gag MA proteins in density gradients (Figure 1A). MEK1/2, through which MAPK is

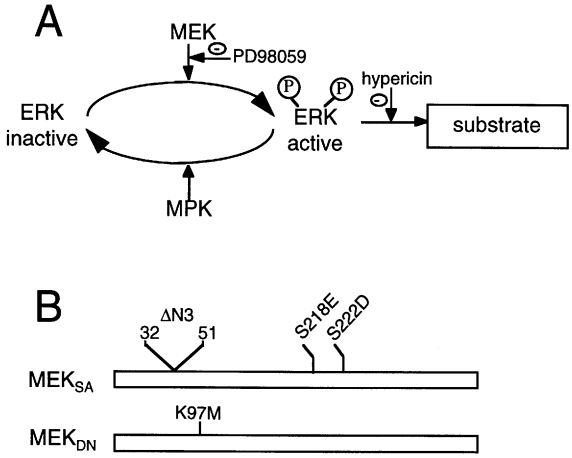

Fig. 2. (**A**) Regulation of ERK activity. ERKs are activated by dual phosphorylation on Thr and Tyr by MEKs (MEK1/MEK2) and inactivated by dephosphorylation of these sites by MAPK phosphatase (MKP). The inhibitor PD98059 binds inactive MEKs, thereby preventing their activation, while Hypericin blocks ERK activity. (**B**) Representation of constitutively active and inactive MEK1 mutants MEK1. Constitutively active MEK1 (MEK_{SA}) carries a deletion from residue 32 to residue 51 (ΔN3) as well as mutations of S218 to E and S222 to D as described elsewhere (Mansour *et al.*, 1994). Mutation of residue K97 to M results in a catalytically inactive MEK1 (MEK_{DN}).

activated (Marshall, 1994; Cobb and Goldsmith, 1995), was not found to associate with virions in sucrose density gradients (Figure 1A). In addition, antibodies to JNK/SAPK cascade components (PAK 65, JNK) or to the p38/HOG cascade (p38) did not reveal detectable quantities of these proteins in association with purified HIV-1 virions (Figure 1A). Moreover, antibodies to p38 MAPK and p65 PAK failed to immunoprecipitate any kinase activity from sucrose gradient-purified HIV-1 virions (not shown), providing additional evidence that these kinases did not associate with HIV-1 virions. ERK/MAPK also associated with virions obtained from peripheral blood mononuclear cells (PBMCs) (not shown). ERK/MAPK was not detectable in sucrose density gradient-fractionated supernatants obtained from uninfected MT-4 cells (Figure 1B). These results indicate selective association of ERK/MAPK with virions of HIV-1.

Modulation of virion-associated ERK/MAPK activity in trans

We next examined whether virion-associated ERK/MAPK retained kinase activity and whether this activity could be modulated *in trans* following stimulation of MAPK activity in the virus-producing cell. In the MAPK cascade, MEK1/2 are the only known activators of ERK1/2 (Figure 2A) and are believed to phosphorylate only these two substrates (Robinson *et al.*, 1996). 293T cells (Pear *et al.*, 1993) were co-transfected with $pHIV_{ELI}$, an infectious molecular clone which is intact for all HIV-1 open reading frames (Peden *et al.*, 1991) and with vectors which express human ERK2/MAPK (Seth *et al.*, 1992), a constitutively active MEK1 (MEK_{SA}) or a kinase-inactive MEK1 (MEK_{DN}) (Mansour *et al.*, 1994) (Figure 2B). HIV-1 virions were harvested from supernatants of co-transfected 293T cells and virus yield determined from RT activity (Figure 3A). Virions were fractionated on sucrose density gradients and kinase activity in individual gradient frac-

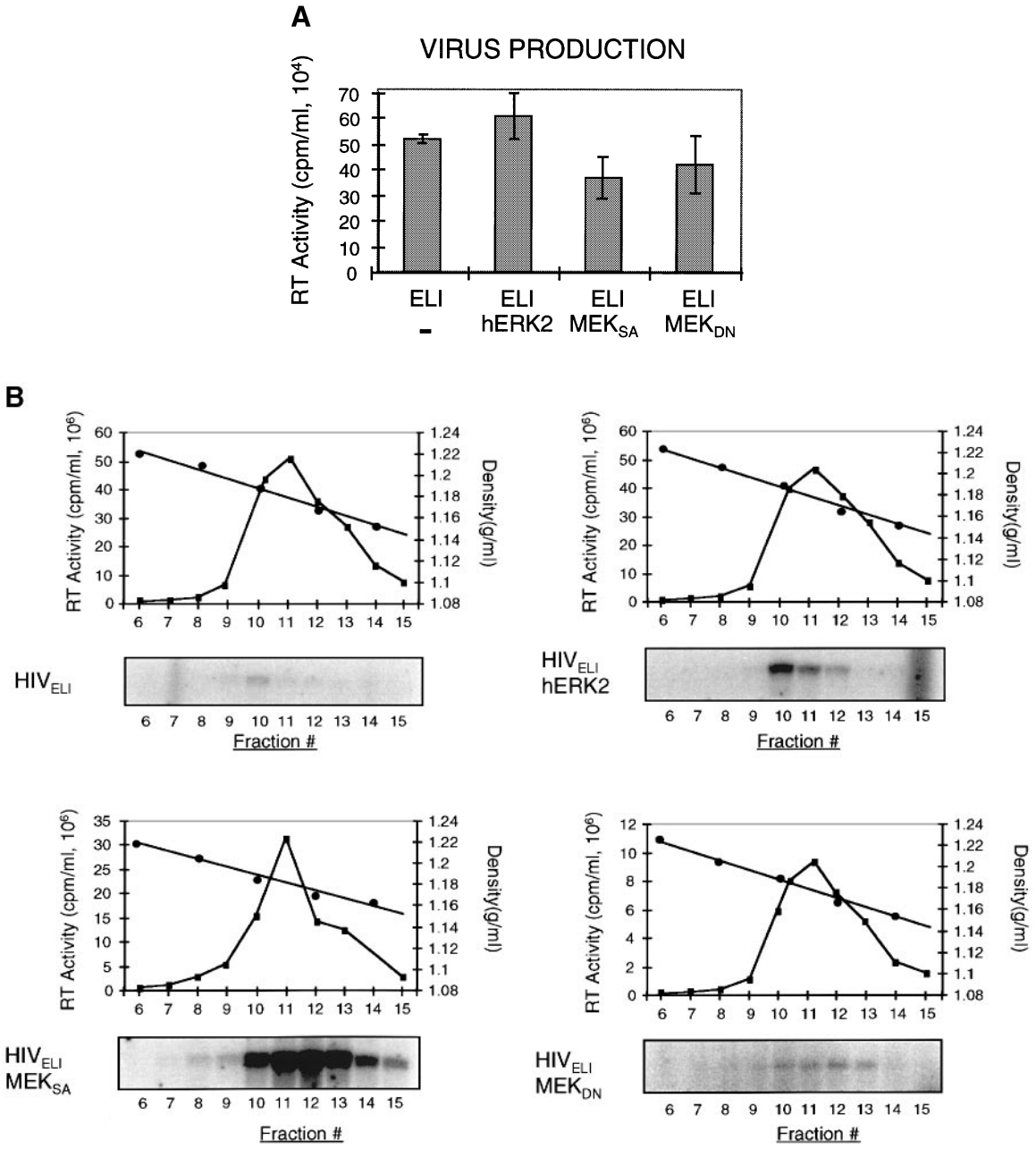

Fig. 3. Modulation of virion-associated ERK/MAPK activity *in trans*. (**A**) 293T cells were transfected with HIV_{ELI}, an infectious molecular clone of HIV-1, or co-transfected with HIV_{ELI} and with vectors which express human MAPK/ERK (hERK2), constitutively active MEK1 (MEK_{SA}) or a non-activating mutant of MEK1 (MEK_{DN}). Virus yield in transfected culture supernatants was determined by RT activity. (**B**) Virions released from transfected 293T cells were subsequently purified, resolved on sucrose density gradients and the levels of virion-associated kinase activity in each gradient fraction was determined in an *in vitro* kinase assay following the addition of MBP (1 μg) as an exogenous substrate. *In vitro* kinase assays were performed as detailed in Materials and methods. Phosphorylated MBP was resolved by SDS–PAGE and visualized on a molecular PhosphorImager.

tions examined in an *in vitro* kinase assay with myelin basic protein (MBP), a preferred MAPK substrate (Figure 3B). Co-expression of ERK2/MAPK increased the relative activity of the virion-associated kinase (Figure 3B) in those fractions containing virion proteins. In contrast, co-transfection of cells with an HIV molecular clone and either p65 PAK or MEK expression plasmids did not result in incorporation of either p65 PAK or MEK into virions (not shown). HIV-1 virions from cells which co-expressed a constitutively active MEK (MEK_{SA}) exhibited a marked increase in virion-associated kinase activity (Figure 3B), but this level of activation was not observed when virions were produced from cells expressing the inactive MEK_{DN} mutant (Figure 3B). Expression of ERK2/MAPK or MEK1 in the virus producer cell did not affect the extent of virus production from co-transfected cells (Figure 3A). Taken together, these results suggest that incorporation of ERK/MAPK into HIV-1 virions and the activation state of ERK/MAPK in the virion can be modulated *in trans*.

Cellular microvesicles have been reported to be a contaminating component of purified virion preparations (Bess *et al.*, 1997; Gluschankoff *et al.*, 1997). In addition, the study by Bess *et al.* (1997) demonstrated that, following

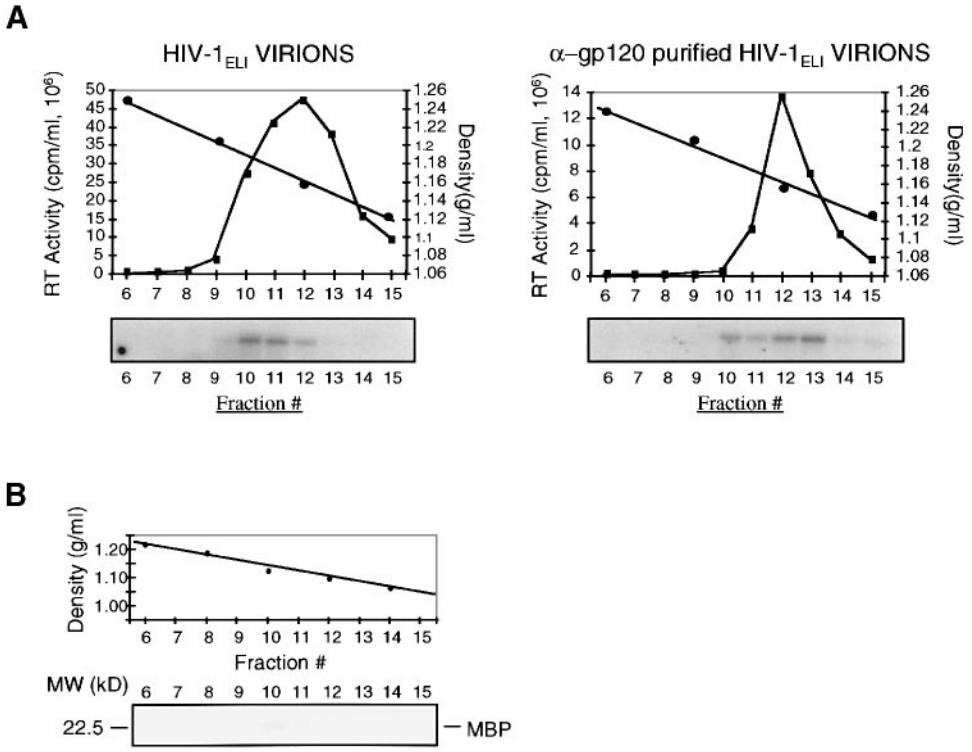

Fig. 4. Virion-associated kinase activity in immunoaffinity-purified HIV-1 virions. (**A**) Virion-associated kinase activity (MBP substrate) in gradient-fractionated virions (left panel) and in immunoaffinity-purified and gradient fractionated virions (right panel) was examined as outlined in Figure 3. (**B**) Culture supernatants of uninfected MT-4 cells were fractionated on sucrose density gradients as for infected culture supernatants and gradient fractions examined for the presence of kinase activity (MBP substrate).

purification of virions by sucrose density gradient centrifugation, most HIV-1 gp120 was found in parallel with gag CA and not with cellular proteins, indicating that little or no gp120 was associated with microvesicles. Therefore, in order to exclude the possibility that kinase activity in HIV-1 virions was due to the presence of contaminating cellular material in density gradients, we further immunoaffinity-purified HIV-1 virions with envelope-specific (anti-gp120) antibodies. Immunoaffinity-purified virions were then fractionated on sucrose density gradients and analyzed for virion-associated kinase activity. Immunoaffinity-purified virions still exhibited virion-associated kinase activity, indicating that the kinase was associated physically with HIV-1 virions (Figure 4A). In addition, there was almost no detectable kinase activity in gradient fractions from uninfected culture supernatants (Figure 4B), indicating that the observed kinase activity, which associated with HIV-1 virions, was not due to the presence of contaminating cellular microvesicles in virion preparations.

HIV-1 gag MA is a substrate for virion-associated ERK/MAPK

Recent studies have suggested that the structural virion protein, gag MA, is a predominant substrate for the virion-associated serine/threonine kinase (Bukrinskaya *et al.*, 1996). Thus, we examined whether phosphorylation of virion-associated gag MA was affected *in trans* by stimulation or inhibition of the MAPK pathway. The mitogen phorbol myristate acetate (PMA) was used to activate the MAPK pathway (Fairhurst *et al.*, 1993), while the compounds PD98059 (2′-amino-3′-methoxyflavone) and Hypericin were used to inhibit the action of MEK and ERK/MAPK, respectively. PD98059 has a high degree of specificity for MEK1 (Alessi *et al.*, 1995; Dudley *et al.*, 1995). Hypericin exhibits IC_{50}s of 4 nM and 3.3 μM for MAPK and PKC, respectively (Takahashi *et al.*, 1989; Agostinis *et al.*, 1995). Thus, we used Hypericin at a concentration (100 nM) which was expected to affect only MAPK activity. $HIV\text{-}1_{ELI}$ virions were harvested from productively infected MT-4 cells following treatment with PMA alone or PMA with either of the MAPK inhibitors. Virions were purified by sucrose density gradient centrifugation, and phosphorylation of virion proteins was examined in an endogenous kinase assay in which virion proteins serve as substrates for the virion-associated kinase (Bukrinskaya *et al.*, 1996). In this endogenous kinase assay, gag MA was the predominant substrate of the virion-associated kinase (Figure 5). There are two phosphorylated species of gag MA which are more clearly identifiable in gradients of PMA-treated virions (Figure 5). We have reported previously (Bukrinskaya *et al.*, 1996) that both phosphorylated MA species are specifically immunoprecipitated by gag MA antibodies and likely represent differentially phosphorylated gag MA proteins. Phosphorylation of the stoichiometrically equivalent structural gag CA protein was less apparent in these endogenous reactions when compared with gag MA (Figure 5). Phosphoamino acid analysis confirmed that phosphorylation of gag MA occurred primarily on serine and, to a lesser extent, on threonine but not on tyrosine (not shown), in agreement with our previous observations (Bukrinskaya *et al.*, 1996). Kinase activity was apparent only upon disruption of virions through treatment with Nonidet P-40 followed by

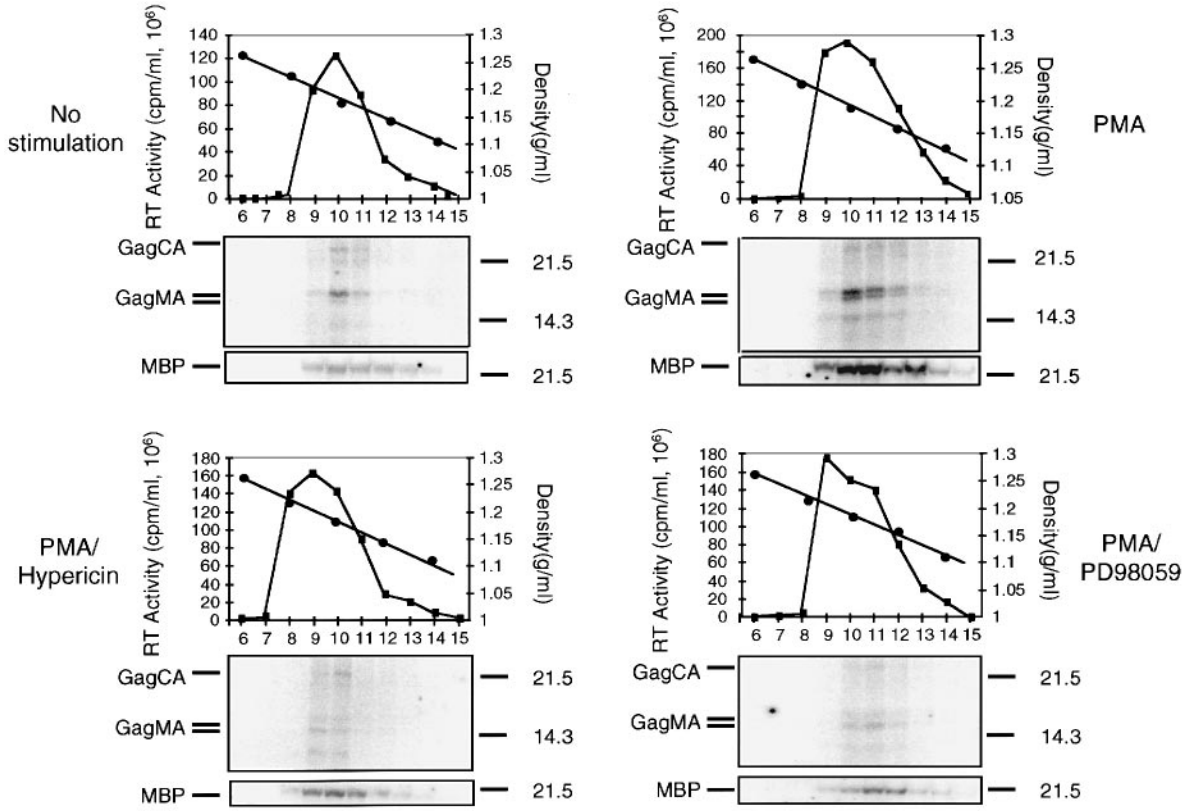

Fig. 5. Modulation of the MAPK pathway affects virion-associated kinase activity. HIV-1-infected MT4 cells were incubated in the presence of PMA or PMA plus the indicated MAPK kinase inhibitors. At 15 h after incubation, virions were harvested from culture supernatants, purified and resolved on sucrose density gradients. Virion-associated kinase activity was evaluated in an endogenous kinase reaction in which virion proteins serve as kinase substrates. In addition, virion-associated kinase activity with MBP as a substrate was evaluated in parallel.

freeze–thaw disruption (not shown). PMA stimulation led to a significant increase in the phosphorylation of virion-associated gag MA protein (Figure 5) and a corresponding increase in the phosphorylation of MBP (Figure 5). PMA stimulation did not influence phosphorylation of gag CA protein. Conversely, inhibition of MEK and MAPK by PD98059 and Hypericin, respectively, led to a significant reduction in gag MA phosphorylation but not gag CA phosphorylation in the endogenous kinase reaction (Figure 5). A phosphoprotein of ~15 kDa was detectable in endogenous kinase reactions. This protein did not react with any HIV-specific gag antibodies, nor was its phosphorylation influenced by PMA or MAPK inhibitors. Although PD98059 and Hypericin reduced phosphorylation of gag MA to almost undetectable levels, we observed a lesser effect on kinase activity using MBP as a substrate (Figure 5). It is possible that kinases in addition to MAPK are contained within HIV-1 virions and that their activity is less sensitive to inhibitors of the MAP kinase pathway. However, the data also suggest that these kinases did not use gag MA as a substrate. Alternatively, inhibitors of the MAP kinase pathway may more efficiently prevent phosphorylation of a gag MA substrate than an MBP substrate.

We demonstrated (Figure 3) that virus particles derived from cells which were co-transfected with an infectious HIV-1 molecular clone and a vector which expresses a constitutively active MEK had increased levels of virion-associated kinase activity when an exogenous MBP was used as a substrate. Therefore, we determined whether the presence of MEK influenced the ability of virion-associated MAPK to phosphorylate the endogenous virion-associated gag MA protein. 293T cells were transfected with the infectious HIV-1 molecular clone $pHIV_{ELI}$ either alone or with vectors which expressed constitutively active MEK1 (MEK_{SA}) or a kinase-inactive MEK1 (MEK_{DN}) as outlined in Figure 3. When compared with virions transfected with the HIV-1 clone alone (HIV_{ELI}), virion-associated kinase activity on gag MA protein was markedly increased in virions obtained from cells which had expressed MEK_{SA} (Figure 6). In contrast, kinase activity on gag MA protein was slightly decreased when virions were obtained from cells which expressed the kinase-inactive MEK_{DN} (Figure 6). This provides further evidence that gag MA is a substrate for virion-associated ERK/MAPK and that the phosphorylation of gag MA in the virion can be influenced *in trans* by modifying the activity of ERK/MAPK in the virus producer cell.

Direct evidence for the phosphorylation of gag MA by ERK/MAPK was obtained following immunoprecipitation of ERK/MAPK from cell lysates by an ERK/MAPK-specific antibody (C14). Immunoaffinity-purified ERK/MAPK was able directly to phosphorylate recombinant gag MA (Figure 7A) or gag MA in purified virion lysates (Figure 7B). In addition, a GST–ERK2 fusion protein was also able to phosphorylate recombinant gag MA *in vitro* (Figure 7A). Treatment of ERK/MAPK immunoprecipitates with MAPK phosphatase (MKP) (Sun *et al.*, 1993; Ward *et al.*, 1994) impaired its phosphorylation of recombinant gag MA (Figure 7A). In addition, MAPK

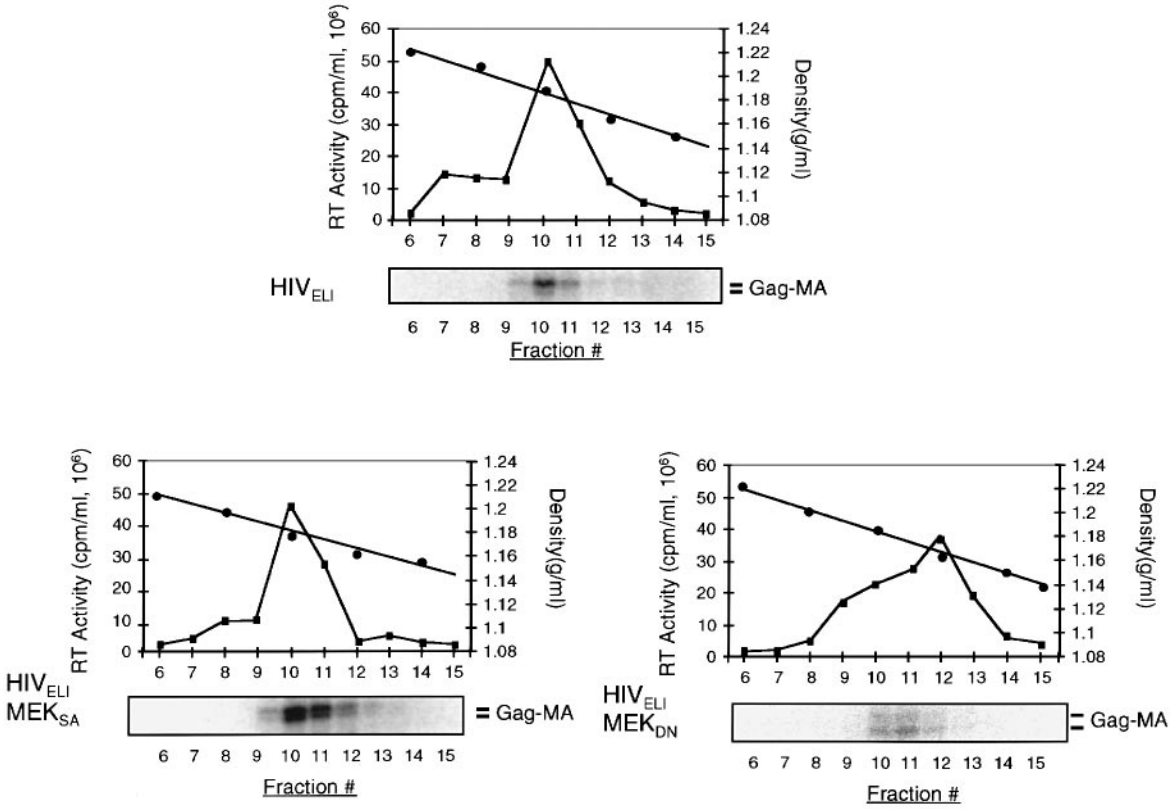

Fig. 6. Modulation of the MAPK pathway influences gag MA phosphorylation. 293T cells were transfected with HIV_{ELI} alone or with vectors which express a constitutively active MEK1 (MEK_{SA}) or a non-activating mutant of MEK1 (MEK_{DN}) as outlined in Figure 3. Virions released from transfected cells were resolved on sucrose density gradients and the ability of the virion-associated kinase to phosphorylate endogenous virion gag MA protein in each fraction was determined in an *in vitro* kinase assay.

phosphatase reduced the phosphorylation of recombinant gag MA and MBP by the virion-associated kinase (Figure 7C). Collectively, these results indicate that gag MA is a MAPK substrate. Although immunoaffinity-purified ERK/MAPK phosphorylated gag CA (24 kDa protein) in virion lysates (Figure 7b), gag CA was not phosphorylated in endogenous kinase assays using purified virions (Figure 5). The preferential phosphorylation of gag MA in these kinase reactions suggests that gag MA and the virion-associated kinase may be co-localized in the virion and that gag CA is inaccessible to the virion-associated kinase. This may also suggest that association with gag MA provides a mechanism for virion incorporation of ERK/MAPK.

We next conducted phosphopeptide mapping of gag MA which had been phosphorylated by virion-associated kinase and by recombinant ERK2/MAPK. Virion-associated gag MA was obtained from lysates of gradient-purified HIV-1 virions which had been immunodepleted of endogenous kinase activity with an antibody to either ERK2/MAPK or p38 MAPK. Gag MA was then phosphorylated *in vitro* following addition of recombinant ERK2/MAPK. Antibodies to ERK2/MAPK but not p38 MAPK were able completely to deplete kinase activity from virion lysates (Figure 8A). Kinase activity was restored following addition of recombinant ERK2 to the ERK2-depleted lysates (Figure 8A). Phosphopeptide maps were generated for gag MA from ERK2-immunodepleted virion lysates which had been phosphorylated *in vitro* by

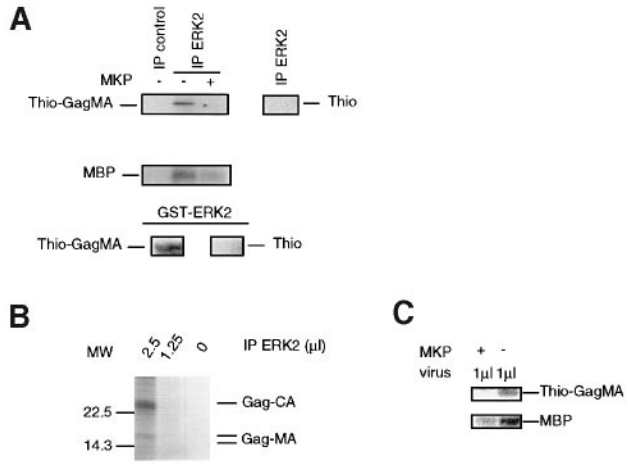

Fig. 7. HIV-1 gag MA is a MAPK substrate. (**A**) ERK/MAPK was immunoaffinity-purified from uninfected MT-4 cells and activity was examined in an *in vitro* kinase assay using a recombinant thioredoxin–gag MA fusion protein (Thio–gagMA), thioredoxin (Thio) or MBP as substrates. Kinase activity was further evaluated following treatment of ERK/MAPK immunoprecipitates with MAPK phosphatase (MKP) (1 µg, 30 min, 37°C). Kinase activity in MAPK phosphatase-treated ERK/MAPK immunoprecipitates was then examined using MBP as a substrate. (**B**) Phosphorylation of virion proteins by immunoaffinity-purified ERK/MAPK. (**C**) MKP was added to virion lysates. Virion-associated kinase activity was then examined in an *in vitro* kinase assay following addition of recombinant gag MA or MBP as kinase substrates.

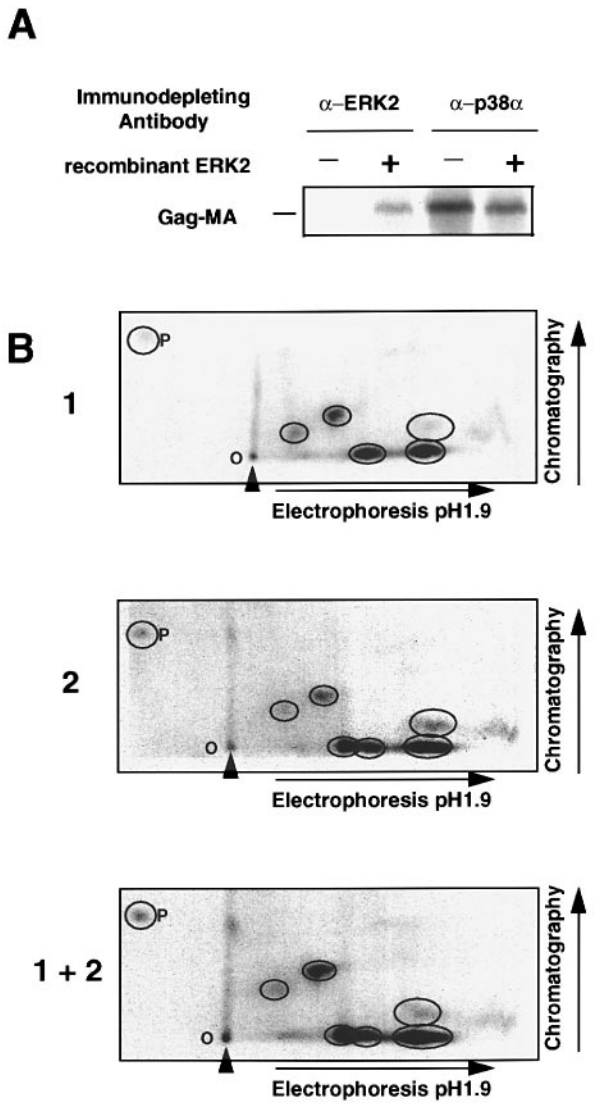

Fig. 8. Phosphopeptide analysis of ERK/MAPK-phosphorylated gag MA. (**A**) Lysates of gradient-purified HIV-1 virions were immunodepleted with antibodies to ERK2/MAPK or to p38 MAPK. Gag MA in immunodepleted lysates was then phosphorylated in the presence or absence of recombinant ERK2/MAPK. (**B**) Two-dimensional phosphopeptide analysis of gag MA from anti-ERK2 antibody-depleted virion lysates which had been phosphorylated *in vitro* by recombinant ERK2/MAPK (panel 1) and of gag MA from anti-p38 MAPK antibody-depleted virion lysates which had been phosphorylated by endogenous virion-associated ERK/MAPK (panel 2). Phosphopeptides shown in panels 1 and 2 were mixed and resolved in two dimensions (panel 1+2).

recombinant ERK2 (Figure 8B, panel 1). This map revealed five major phosphopeptides and was almost identical to the phosphopeptide pattern obtained from gag MA in p38 MAPK-immunodepleted virion lysates which had been phosphorylated by the endogenous virion-associated kinase (Figure 8B, panel 2). Phosphopeptides from gag MA phosphorylated by recombinant ERK2 and by virion-associated kinase were mixed and five out of six phosphopeptides were identical (Figure 8B, panel 1+2). One phosphopeptide was unique to gag MA that had been phosphorylated by the virion-associated kinase. Collectively, this suggests that similar phosphate acceptor sites on gag MA protein are utilized by virion-associated and recombinant ERK2/MAPK.

ERK/MAPK regulates HIV-1 infectivity

We next determined whether modulation of virion-associated ERK/MAPK activity influenced viral infectivity. Virus particles produced in the presence of PMA, PD98059 and Hypericin were purified and virion infectivity was examined in a single cycle transactivation-based assay which provides a quantitative measure of the efficiency of provirus establishment in the target cell (Kimpton and Emerman, 1992). Stimulation of virion-associated kinase activity and gag MA phosphorylation by PMA correlated with a significant increase (2- to 4-fold) in viral infectivity (Figure 9A). This effect on viral infectivity was not due to the effects of PMA on late events in the virus life cycle (those events which follow transcriptional activation of the provirus) since PMA did not affect the levels of virus production (Figure 9B). Conversely, PD98059 and Hypericin both significantly impaired the infectivity of virions which had been derived from PMA-induced cells (Figure 9A) but did not influence virus production (Figure 9B). While both PD98059 and Hypericin exhibited similar inhibitory activities on infectivity of viruses produced in the absence of PMA, PD98059 appeared to be less effective than Hypericin in blocking the infectivity of viruses produced in the presence of PMA. However, the degree of inhibition as a percentage of the non-treated control is similar. Thus, for viruses produced in the absence of PMA, PD98059 reduced virus infectivity by ~5-fold and for virions produced in the presence of PMA infectivity was also reduced by ~5-fold by PD98059. We should point out that PD98059 did not completely block gag MA phosphorylation in virions obtained from PMA-treated cells (Figure 5). Thus, the effect of PD98059 on virus infectivity parallels its effects on gag MA phosphorylation. Addition of PD98059 or Hypericin to target cells 1 h after virus infection did not impair viral infectivity (not shown). In addition, since the infectivity assay is conducted under single-cycle conditions (without successive rounds of virus replication) and since neither PMA, PD98059 nor Hypericin influenced virus production (Figure 9B), the results suggest, collectively, that MAPK regulates an early step in the virus life cycle.

We attempted to modulate HIV infectivity more directly by examining the single-cycle infectivity of viruses derived from cells which express a kinase mutant variant of MEK (MEK_{DN}). For these experiments, COS7 cells were co-transfected with the infectious HIV-1 molecular clone, HIV-1_{ELI} together with a vector which expresses a constitutively active MEK (MEK_{SA}) or a dominant-negative mutant of MEK (MEK_{DN}). At 24 and 48 h post-transfection, viruses were harvested from supernatants of transfected COS7 cells and normalized according to virion-associated RT activity. Infectivity of viruses was examined in a single-cycle transactivation-based assay at four different multiplicities of infection. Infectivity of viruses produced from cells which expressed a mutant MEK but not an active MEK were reduced 4-fold (Figure 10A) [compare the amount of MEK_{DN} virus needed for equivalent infectivity as the control; thus, 0.125 and 0.25 units (1 unit = 10^6 c.p.m. reverse transcriptase activity) of control virions produce the same level of infection as 0.5

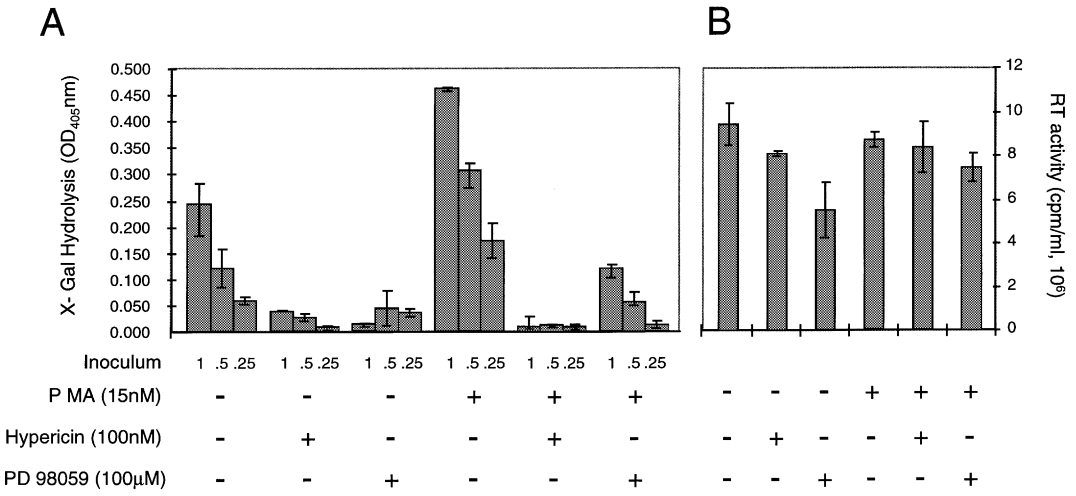

Fig. 9. ERK/MAPK modulates HIV-1 infectivity. HIV-1 infected MT-4 cells were treated with the indicated MAPK inhibitors in either the presence or absence of PMA for 15 h. (**A**) Infectivity of HIV-1 virions was evaluated at three different levels of input virus in a single-cycle transactivation-based assay (Kimpton and Emerman, 1992). HIV-1 tat-mediated transactivation (as determined from hydrolysis of X-gal) β-gal production was quantitated at 24 h post infection (mean ± SD, $n = 3$). (**B**) Modulation of MAPK activity does not influence virus production. The extent of virus production following treatment of HIV-1-infected MT-4 cells with MAPK modulators was determined from virion-associated RT activity in culture supernatants.

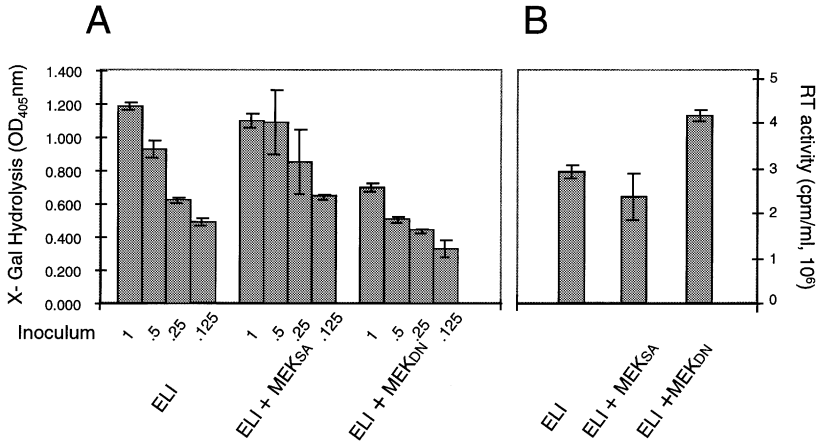

Fig. 10. A dominant-negative form of MEK impairs HIV-1 infectivity. (**A**) COS7 cells were co-transfected with HIV_{ELI}, an infectious molecular clone of HIV-1, or co-transfected with HIV_{ELI} and with vectors which express constitutively active MEK1 (MEK_{SA}) or a non-activating mutant of MEK1 (MEK_{DN}). Virions released from transfected COS7 cells were harvested at 24 and 48 h post-transfection and virion infectivity was evaluated at four different levels of input virus in a single-cycle transactivation-based assay ($n = 5$) as outlined in Figure 9. Levels of virus produced from transfected COS7 cells (**B**) was determined from virion-associated RT activity in culture supernatants at 48 h post-transfection ($n = 5$).

and 1 units of MEK_{DN} virus, respectively]. This inhibition was markedly consistent over five independent experiments. In contrast, there was an augmentation in infectivity of viruses produced from cells which expressed a constitutively active MEK (Figure 10A). The impaired infectivity of viruses produced from cells which express MEK_{DN} was independent of any effects on virus production (Figure 10B). These studies further support the notion that HIV infectivity is modulated by the ERK/MAPK kinase cascade.

ERK/MAPK is required for establishment of a functional reverse transcription complex

To characterize further the stage in HIV-1 infectivity which was being impaired by MAPK inhibitors, we examined synthesis of viral nucleic acids in infected target cells by PCR. Immediately following entry of viral nucleic acids into the target cell, minus-strand strong stop cDNA is the first detectable product of reverse transcription. HeLa-CD4-LTR/β-gal cells were infected with viruses that had been produced in the presence or absence of the MAPK inhibitors, Hypericin and PD98059. To remove pre-existing viral cDNA in the inoculum, virus preparations were treated with DNase (Zack *et al.*, 1990). Cells were infected in the presence of 3′-azido-3′-deoxythymidine (AZT, 5 μM) in order to evaluate *de novo* synthesis of viral cDNA in the target cell (Heinzinger *et al.*, 1994). This concentration was sufficient to eliminate completely *de novo* synthesis of viral cDNA. Although *de novo* synthesis of strong stop cDNA by MAPK-inhibitor-treated virions was evident (Figure 11, compare quantities of PCR products in AZT-treated and untreated panels), the levels of strong stop cDNA were reduced relative to control cultures for both Hypericin- and PD98059-treated virions (Figure 11). In addition, where levels of strong stop cDNA products increased in control cultures, there was a

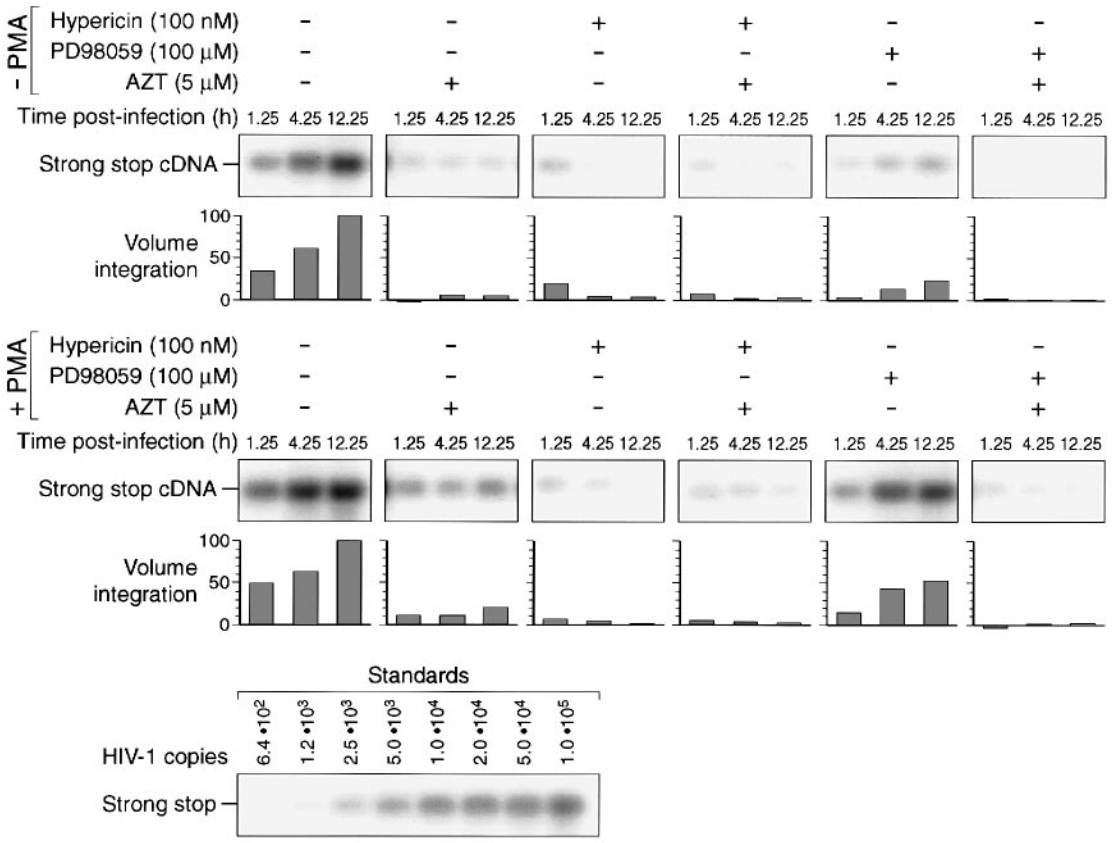

Fig. 11. ERK/MAPK is required for establishment of a functional reverse transcription complex in the target cell. HIV-1-infected MT-4 cells were cultured in the presence or absence of PMA and MAPK inhibitors and virions were harvested after 15 h, pelleted and resuspended in PMA/inhibitor-free medium and treated with DNase to remove carryover DNA in the inoculum (Zack *et al.*, 1990). Viral supernatants were normalized for infection of HeLa-CD4-LTR/β-gal cells (Kimpton and Emerman, 1992) at three multiplicities of infection. At the indicated times post infection, cell aliquots were removed for analysis of minus-strand strong stop cDNA by PCR. Infections carried out in the presence of AZT (5 μM) were used to verify *de novo* synthesis of viral cDNA. Copy number standards were generated by PCR on serial 2-fold dilutions of 8E5 cells, containing a single defective provirus per cell (Folks *et al.*, 1986).

corresponding decrease in the abundance of minus- strand strong stop cDNA in cells infected with Hypericin-treated virions (Figure 11). This suggested either that the genomic viral RNA precursor of the minus-strand strong stop cDNA was unstable, or alternatively that the stability of the reverse transcription complex itself was compromised. We also examined for the presence of late products (U5/gag) in reverse transcription, namely those which are formed following the second template switch (Bukrinsky *et al.*, 1993b). Late products of reverse transcription were not synthesized in cells which had been infected with Hypericin-treated virions and were markedly reduced in cells infected with PD98059-treated virions (not shown). We do not believe that the kinase inhibitors were influencing reverse transcription through impairment of Vif phosphorylation, as the target cells used for virus infection in this study do not require Vif to support virus replication.

We demonstrated previously that inhibition of gag MA phosphorylation impairs its efficient translocation to the nucleus and concomitantly increases the amount of gag MA that is present in the membrane of the infected cell (Boukrinskaya *et al.*, 1996). Therefore, we examined whether inhibitors of the ERK/MAPK pathway also altered the subcellular distribution of gag MA in the infected cell. HIV-1 gag MA is co-translationally myristoylated in the virus life cycle. Thus, HIV-1 virions were obtained from cells which had been cultured in the presence of [^{3}H]myristic acid. To inhibit virion-associated kinase activity, virus-producing cells were incubated with either Hypericin or PD98059. MT-4 cells were then infected in the absence of the MAPK inhibitors and at 3 h post-infection, membrane/cytosol and nuclear extracts were prepared and the distribution of ^{3}H-labeled gag MA in these fractions was determined (Figure 12). We have demonstrated previously (Boukrinskaya *et al.*, 1996) that the majority (95%) of gag MA in the infected cell is present in the membrane/cytosol fraction, while ~5% of gag MA in the infected cell localizes to the host cell nucleus. In addition, gag MA is the only virion protein to incorporate [^{3}H]myristic acid. Levels of gag MA in the nucleus were reduced to 57 and 76% of control levels when viruses were prepared in the presence of Hypericin and PD98059, respectively (Figure 12). There was a concomitant increase in the relative amount of ^{3}H-labeled gag MA in the membrane/cytosol fraction. The total amount of gag MA in the infected cell was not affected by the MAPK inhibitors, indicating that these agents did not impede the ability of the virions to enter the target cell, but rather affected a post-entry step. Thus, in agreement with our previously published study (Boukrinskaya *et al.*, 1996), these data indicate that phosphorylation of gag MA by ERK/MAPK ultimately influences its subcellular distribution in the

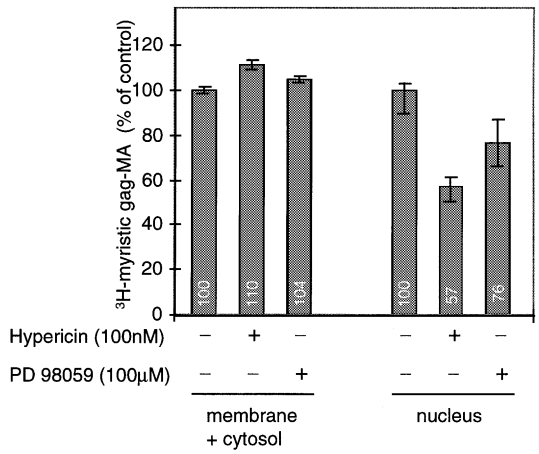

Fig. 12. Virion-associated ERK/MAPK influences the subcellular distribution of myristoylated HIV-1 gag MA following acute infection. HIV-1-infected MT-4 cells were incubated in the presence of [^{3}H]myristic acid (10 μCi/ml) in the presence or absence of the indicated agents. Virions were harvested and used to infect MT-4 cells and at 6 h post infection, cells were harvested for preparation of membrane/cytosol and nuclear fractions. The distribution of ^{3}H-labeled gag MA was determined from the amount of radioactivity in the cell fractions. Gag MA distribution ($n = 3$) was expressed as a percentage of the level of radioactivity in fractions from cells infected with untreated virions.

target cell. As a consequence, inhibition of ERK/MAPK activity in the virion impairs the relative ability of gag MA to dissociate from the membrane and translocate to the nucleus. Taken together, these data suggest that the activity of virion-associated ERK/MAPK is necessary for the establishment of a functional reverse transcription complex within the target cell.

Discussion

The results outlined in this study indicate that ERK/MAPK associates with HIV-1 virions, phosphorylates the structural virion protein, gag MA, and modulates viral infectivity at a point preceding transcriptional activation of the provirus. During preparation of this manuscript, Cartier *et al.* (1997) demonstrated association of ERK/MAPK with virions of HIV-1, although the biological significance of this observation was not evaluated. Following virus infection, gag MA proteins derived from the virion remain associated with the viral reverse transcription complex as it translocates to the host cell nucleus (Bukrinsky *et al.*, 1993a; Karageorgos *et al.*, 1993; von Schwedler *et al.*, 1994; Gallay *et al.*, 1995; Farnet and Bushman, 1997). During virus assembly, gag MA myristoylation promotes membrane localization of gag polyproteins for incorporation into maturing virions (Göttlinger *et al.*, 1989; Bryant and Ratner, 1990; Gheysen *et al.*, 1990). We demonstrated previously that phosphorylation of gag MA by a virion-associated serine/threonine kinase promotes dissociation (often referred to as uncoating) of the reverse transcription complex from the host cell membrane (Bukrinskaya *et al.*, 1996). We proposed that this membrane dissociation places the reverse transcription complex in a cellular compartment from which the complex can subsequently translocate to the host cell nucleus. The studies outlined here implicate ERK/MAPK as the virion-associated kinase which regulates HIV infectivity, perhaps through phosphorylation of gag MA and facilitation of the uncoating step. Thus, it is likely that MAPK inhibitors block HIV infection by interfering with membrane dissociation of the reverse transcription complex, thereby restricting subsequent translocation of the complex to the host cell nucleus.

At present, it is unclear whether there is a specific mechanism through which ERK/MAPK is incorporated into virions. Upstream activators of ERK/MAPK did not appear to associate with HIV-1 virions. Similarly, overexpression of PAK kinase—a component of the JNK/SAPK cascade—was not sufficient to facilitate association of PAK with the virion or to modulate kinase activity within the virion. Analysis of cellular proteins which may associate with virions is complicated by the presence of cellular microvesicles which frequently contaminate virion preparations (Bess *et al.*, 1997; Gluschankoff *et al.*, 1997). We have adopted preclarification procedures which are effective in removing most of the contaminating cellular material from virion preparations (A.Mann, J.M.Jacqué and M.Stevenson, in preparation). Cellular microvesicles which may remain following less rigorous virion purification may explain the presence of tyrosine kinase activity in HIV-1 virions reported elsewhere (Gallay *et al.*, 1995), since we were unable to detect any tyrosine kinase activity in our virion preparations. It is possible that ERK/MAPK is incorporated specifically into virions through association with its gag MA substrate.

ERK/MAPK is a proline-directed kinase (Davis, 1993). There are no consensus ERK/MAPK recognition sites within gag MA protein of HIV-1. Despite this, ERK/MAPK immunoprecipitates were able to phosphorylate gag MA proteins *in vitro* and this activity was sensitive to MAPK phosphatase. In addition, phosphopeptide maps of gag MA phosphorylated by recombinant ERK2/MAPK and by virion-associated ERK/MAPK were almost identical, indicating that gag MA is an ERK2/MAPK substrate. ERK/MAPK phosphorylation of a protein which lacks MAPK consensus recognition sites has recently been reported (Corbalan-Garcia *et al.*, 1996), and in this case it has been suggested that conformation of the substrate protein was sufficient to allow recognition by MAPK.

Recent studies have demonstrated that the Nef protein of HIV-1 interacts with cellular kinases (Sawai *et al.*, 1994), that Nef is a virion-associated protein (Pandori *et al.*, 1996) and that Nef promotes serine phosphorylation of gag MA (Swingler *et al.*, 1997). We were unable to demonstrate any effects of Nef either on virion association of ERK/MAPK or on the active state of ERK/MAPK within the virion. While our studies fail directly to implicate Nef in promoting association or activation of ERK/MAPK with virions of HIV-1, recent studies from our group (J.-M.Jacqué and M.Stevenson, in preparation) suggest that Nef may activate upstream Src-family kinases which themselves influence activation of the MAPK cascade.

Materials and methods

Transfections and generation of viral stocks

293T cells (Pear *et al.*, 1993) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS). For

generation of viral stocks, 293T cells were transfected with HIV-1_{ELI} proviral DNAs (25 μg) using a modified calcium phosphate/DNA precipitation method (Gibco-BRL) and incubated at 37°C. Virions in culture supernatants were collected at 24 and 48 h post-transfection and either used directly for infection or amplified in MT-4 T cells or in phytohemagglutinin (PHA)-stimulated human PBMCs in complete T-cell medium containing RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. For examination of the effects of MEK_{DN} and MEK_{SA} on HIV-1 infectivity, COS7 cells were transfected with HIV-1_{Eli} and the respective MEK expression plasmid and maintained exactly as detailed above except that cells were cultured in 2% FBS. Virions were harvested at 24 and 48 h post-transfection.

Virus purification

Supernatants from transfected 293T cells or from infected PBMCs or MT-4 cells were cleared of cellular debris by low-speed centrifugation (1500 *g*, 10 min) and then filtered (0.22 μm). Virions in clarified supernatants were harvested (10 000 *g*, 2 h) and resuspended in serum-free RPMI at 0.005× volume. Concentrated virions were applied to a 15–60% (w/v) continuous sucrose gradient and virions were sedimented at 200 000 *g* for 10–15 h. Gradient fractions were collected (0.5 ml aliquots) and the amount of virus in each fraction was measured by RT activity. For immunoaffinity purification of HIV-1 virions, gradient-purified virus particles were incubated for 1 h with 10 μl of a polyclonal anti-gp120 antibody (ABI), 10 μl protein A–biotin (Amersham), 100 μl of streptavidin-Magnesphere particles (Promega) and 1 mg/ml (final concentration) bovine serum albumin. Bound virions were purified over a magnet, washed (2×1 ml) with phosphate-buffered saline (PBS, pH 7.4) and resuspended in 500 μl serum-free RPMI containing 500 pmol/ml biotin. After an additional 1 h incubation, purified virions were fractionated by sucrose density gradient centrifugation and individual gradient fractions examined for virion-associated kinase activity.

Subcellular fractionation

Membrane/cytosol and nuclear fractions of HIV-1-infected MT-4 cells were prepared at 6 h post infection as described elsewhere (Lu *et al.*, 1993). Concentrations of ^{3}H-labeled gag MA in cell fractions were determined by measurement of radioactivity in each fraction.

Western blot analysis

Gradient-purified virions were solubilized in sample buffer (62.5 mM Tris–HCl pH 6.8, 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol). Viral lysates were denatured by boiling and separated on 12% SDS–polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose (Hybond C Extra, Amersham), blocked in 5% milk and incubated with the respective antibody. Bound antibodies were visualized with peroxidase anti-Ig, followed by ECL (Amersham). The MEK1/2 polyclonal and $p90^{rsk}$ monoclonal antibodies were from Transduction Laboratories, ERK2/MAPK(C14) and p65PAK antibodies were from Santa Cruz Biotechnologies, and p38 MAPK antibodies were from New England Biolabs.

In vitro kinase assays

Gradient-purified HIV-1 virions (normalized by RT activity) were permeabilized in a 10 μl reaction mixture containing 18 μCi of [γ-^{32}P]ATP, 0.5% Nonidet P-40 and 10 mM $MgCl_2$ in 50 mM HEPES pH 7.4. For *in vitro* kinase assays, where indicated, MBP (1 μg) was added to each sample, otherwise endogenous virion proteins served as kinase substrates. Following incubation at 30°C for 30 min, the reactions were stopped by addition of SDS sample buffer and heating to 95°C for 5 min. Phosphorylated proteins were separated by SDS–PAGE and visualized on a molecular PhosphorImager (Molecular Dynamics).

Phosphopeptide mapping

Gradient-purified virus particles (~300 μg) were solubilized in 0.25% Nonidet P-40 followed by three freeze–thaw cycles. Lysates were immunodepleted with 2 μg of ERK2/MAPK antibody or P38 MAPK antibody at 4°C for 1 h followed by a 2 h incubation with 25 μl protein A–agarose. Immunodepleted lysates were then incubated with 10 volumes of *in vitro* kinase buffer containing 70 μCi [γ-^{32}P]ATP. For *in vitro* kinase reactions involving recombinant enzyme, 2 μg of recombinant ERK2/MAPK was added.

Gag MA phosphoproteins were resolved on 15% SDS–PAGE gels. The gel fragment containing gag MA was excised and digested with 30 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Boehringer Mannheim) in 300 μl of 0.25% ammonium bicarbonate, pH 8.0. Gag MA phosphopeptides (500 c.p.m., as determined by Cerenkov measurements) were resolved in two dimensions as detailed elsewhere (Boyle *et al.*, 1991). Briefly, phosphopeptides were separated by electrophoresis (60 min/1000 V) using the Hunter thin-layer electrophoresis system (HTLE-7000, CBS Scientific Co.). Chromatography was performed at right-angles to the direction of electrophoresis for 5 h in 1-butanol/pyridine/acetic acid/water (37.5:25:7.5:30). After chromatography, the plates were dried and phosphopeptides visualized on a PhosphorImager.

Infectivity assays

HeLa-CD4-LTR/β-gal indicator cells (Kimpton and Emerman, 1992) were plated in 96-well microtiter plates (2×10^4 cells per well). HIV-1 virions (normalized to RT activity) were added in doubling dilutions to triplicate wells. At 24 and 48 h post infection, cells were harvested for quantitation of β-galactosidase production (Promega).

PCR analysis of HIV-1 strong stop cDNA synthesis

Total cellular DNA was isolated from acutely infected cells using an IsoQuick DNA extraction protocol (Microprobe Corporation, Bothol, Washington). Minus-strand HIV-1 strong stop cDNA products were amplified by PCR using M667 and AA55 primers as described previously (Zack *et al.*, 1990). Viral cDNA was amplified in 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin. After initial denaturation (95°C, 2 min), amplification proceeded for 25 cycles of PCR (95°C, 30 s; 58°C, 30 s; 72°C, 1 min) followed by a final 5 min extension at 72°C. HIV-1 copy number standards were generated by PCR on serial 2-fold dilutions of 8E5 cells (each containing a defective provirus; Folks *et al.*, 1986). PCR products were Southern blotted onto nylon membranes and hybridized with a ^{32}P-labeled probe consisting of a mixture of two oligonucleotides [g^{566}tctgttgtgtgactctggt-3′ and g^{584}taactagagatccctcagac-3′; primers numbered according to the HIV-$1_{HX\ B2}$ sequence (Myers *et al.*, 1993)]. Hybridized PCR products were visualized on a PhosphorImager. Hybridized products were quantitated by volume integration (local median background subtraction was applied) using Molecular Dynamics ImageQuant software.

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