The role of MKK1/2 kinase activity in human cytomegalovirus infection

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Human cytomegalovirus infection of quiescent fibroblasts was found to induce a bi-phasic activation of mitogen-activated protein kinase (MAPK) kinase 1 and 2 (MKK1/2) and two of their downstream targets, extracellular signal regulated kinase 1 and 2 (ERK1/2), as determined by Western blot analysis using phospho-specific antibodies. Treatment of infected fibroblasts with U0126, a potent and specific inhibitor of MKK1/2 kinase activity, completely blocked ERK1/2 activation following HCMV infection without affecting cell viability. Anti-viral studies demonstrate that in the presence of U0126, viral titres are reduced and viral DNA replication is inhibited. In addition, protein levels of two viral early genes that are required for viral DNA replication, UL44 and UL84, are significantly decreased in the presence of U0126. These results suggest that HCMV-mediated activation of MKK1/2 kinase activity enhances virus infectivity by ensuring timely initiation of viral DNA replication, possibly by regulating early gene expression.

Human cytomegalovirus (HCMV) is a ubiquitous, prototypical betaherpesvirus that is present in 60–80% of the human population (Britt & Alford, 1996; Huang & Kowalik, 1993). One hallmark of HCMV infection is activation of numerous host cell proteins and macromolecular synthesis machinery, many of which are required for HCMV to complete its lytic life-cycle. Several reports suggest cellular kinases have an important role in this virus-mediated activation of the host cell. For example, both the mitogen-activated protein kinase (MAPK) p38 and cyclin-dependent kinase 2 (CDK2) are activated following HCMV infection, and inhibition of their kinase activity greatly decreases viral DNA replication (Bresnahan et al., 1997a, b; Johnson et al., 1999, 2000). In addition, broad-range kinase inhibitors such as H7 can inhibit virus-induced signalling (Boyle et al., 1999; Keay & Baldwin, 1996).

The extracellular signal regulate kinase (ERK) is one of three mammalian MAPK pathways which have been identified [reviewed in Dhanasekaran & Reddy (1998); Tibbles & Woodgett (1999); Widmann et al. (1999)]. The core of all MAPK pathways consists of three sequential kinases: MAPK kinase kinase (MKKK), MAPK kinase (MKK) and MAPK (Widmann et al., 1999). In the case of the ERK pathway, the three sequential kinases are Raf (the MKKK), MKK1/2 (the MKKs) and ERK1/2 (the MAPKs) (Seger & Krebs, 1995). Recently, HCMV infection of fibroblasts has been shown to induce activation of ERK1/2 at 10–15 min post-infection (p.i.) (Boyle et al., 1999). Also, fibroblasts that have been stimulated with serum prior to infection maintain ERK1/2 activation longer following removal of serum than uninfected controls (Rodems & Spector, 1998). In addition, ERK1/2 has been shown to phosphorylate the HCMV immediate early 2-86 (IE2-86) protein, and deletion of ERK1/2 phosphorylation sites alters IE2-86 transactivation function (Harel & Alwine, 1998).

Based on these finding, it has been hypothesized that activation of the ERK MAPK pathway enhances HCMV infection. Unfortunately, attempts to demonstrate this using chemical inhibitors have been unsuccessful due at least in part to the low specificity of available inhibitors (Rodems & Spector, 1998). Recently the chemical compound 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene, or U0126, was identified as a potent and specific inhibitor of both MKK1 and MKK2 kinase activity (Favata et al., 1998). This prompted us to first examine MKK1/2 and ERK1/2 activity throughout the course of viral infection, and then determine whether U0126 could inhibit any observed increase(s) in ERK1/2 activity.

Human embryonic lung (HEL) fibroblasts, which are fully permissive for HCMV infection, were grown to confluence in

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minimal essential media (MEM) (Gibco-BRL) supplemented with 10% foetal bovine serum (Gibco-BRL). Since serum is a strong activator of the ERK pathway, fibroblasts were serum-starved for 48 h in MEM prior to infection to prevent complications arising from serum-mediated ERK1/2 activation (Marshall, 1995; Seger & Krebs, 1995). All infections were carried out as previously described using Towne strain HCMV (passage 39–42) that had been purified by ultracentrifugation through a sucrose cushion (Johnson et al., 2000). Where indicated, cells were treated with U0126 (10 µM) for 1 h prior to infection. U0126 was also present during infection and subsequent incubation periods. At the indicated times, cells were harvested in 2× Laemmli SDS sample buffer, and MKK1/2 and ERK1/2 activation was analysed by Western blot analysis using phospho-specific antibodies which only recognize the phosphorylated, and hence active, form of the protein (New England BioLabs). As Fig. 1(A, B) shows, two phases of MKK1/2 and ERK1/2 activation were detected following HCMV infection. The first phase of activation occurred between 10–30 min after addition of virus (Fig. 1 A, compare lane 1 with lanes 2 and 3). This correlates well with a recently published report which showed that binding of the viral glycoprotein gB – located in the virion envelope – to host cell receptors was sufficient to obtain activation of ERK1/2 at immediate early times of infection (Boyle et al., 1999). Thus, the MKK1/2 and ERK1/2 activation observed in Fig. 1(A) is likely induced by binding of gB to the host cell receptors. Between 4 and 12 h p.i., a second increase in both MKK1/2 and ERK1/2 activation was detected (Fig. 1B, compare lanes 2 and 3). While MKK1/2 activation had declined to basal level between 12–24 h p.i., ERK1/2 activity remained elevated until 24–48 h p.i. (lane 4). Previously, Rodems & Spector (1998) reported that in HCMV-infected fibroblasts, ERK1/2 was not activated beyond 8 h p.i. This apparent discrepancy is easily explained by the facts that (1) the majority of their experiments examined the ability of HCMV to maintain ERK1/2 activation in serum-stimulated fibroblasts (as opposed to this study, which examines the ability of HCMV to induce ERK1/2 activation in quiescent fibroblasts); and (2) when Rodems and Spector examined the affect of HCMV on ERK1/2 activation in unstimulated fibroblasts, only early times of infection were examined (up to 8 h).

Finally, Fig. 1(C, D) shows that treatment of infected fibroblasts with 10 µM U0126 strongly inhibited ERK1/2 activation throughout the course of infection. This is the first demonstration that HCMV infection activates MKK1/2 in addition to ERK1/2, and that activation of ERK1/2 is dependent upon MKK1/2 kinase activity throughout the course of infection. These results also suggest that U0126 can completely inhibit MKK1/2 activation of ERK1/2, and therefore can be utilized to determine if inhibiting the ERK pathway kinase activity affects the virus life-cycle.

The observation that the ERK pathway was activated 8–24 h p.i. suggests that it may have a role in initiation of viral DNA replication. To test this, viral DNA replication in the
presence of U0126 was examined by DNA hybridization as previously described (Johnson et al., 1999). Briefly, fibroblasts were grown to confluence in 24-well dishes, serum starved for 48 h in MEM, and then pre-treated with the indicated concentrations of U0126 for 1 h prior to infection with HCMV. Following infection, fibroblasts were maintained in MEM containing the appropriate concentration of U0126. Due to the short half-life of U0126 at 37 °C, the medium (containing the appropriate concentration of U0126) was changed every 48 h. As a positive control, cells were infected in the presence of ganciclovir (DHPG), a potent inhibitor of HCMV DNA replication (Mar et al., 1983). At the indicated times, cells were harvested, total DNA was isolated and the amount of viral DNA determined by dot blot hybridization using purified 32P-labelled HCMV DNA. The results, depicted in Fig. 2(A), show that 1 µM U0126 inhibited viral DNA replication by about 60%, and 10 µM U0126 inhibited viral DNA replication by > 90%. Interestingly, if U0126 was not added until 4 h after infection (after the first phase of MKK1/2 activation was finished), viral DNA replication was inhibited, but not to the same extent as that observed when U0126 was added prior to infection (data not shown). This suggests that both phases of the ERK pathway activation contribute to initiation of viral DNA replication.

To determine if the decrease in viral DNA replication affected viral titres, a titre reduction assay was performed. Cells were grown to confluence, infected, and incubated as for the viral DNA hybridization assay. Six days after infection, the cellular supernatant was harvested and utilized in a standard plaque assay. As Fig. 2(B) shows, the presence of 1 µM U0126 reduced viral progeny by over 70% relative to untreated controls, while 5 and 10 µM U0126 reduced viral progeny by 90 and 99%, respectively.

To show that the antiviral effects of U0126 were not due to cellular toxicity, fibroblasts were first incubated in MEM without serum or phenol red in the presence or absence of 10 µM U0126. After 96 h, the supernatant was harvested and cellular toxicity was determined using the CytoTox assay kit according to manufacturer’s protocol (Promega). The results, depicted in Fig. 2(C), indicate that no significant increase in cytotoxicity was detected in the presence of U0126 relative to untreated controls (compare bars 3 and 4). These results show for the first time that preventing MKK1/2 activity significantly inhibits the virus life-cycle.

Finally, we wanted to determine at least one mechanism by which MKK1/2 kinase activity affects viral DNA replication. It is possible that MKK1/2 kinase activity enhances HCMV viral DNA replication by influencing viral gene expression. IE gene expression initiates 30–60 min p.i., and is dependent upon activation of host cell transcription factors, some of which may be affected by ERK activation (Mocarski, 1996). Western blot analysis demonstrated that in the presence of U0126, protein levels of the two major IE genes, IE1-72 and IE2-86, were slightly decreased at 8 h p.i., though substantial IE1-72 and IE2-86 protein was still detected (Fig. 3 A). In addition, by 24 h p.i., IE1-72 and IE2-86 proteins levels were the same between the treated and untreated cells (Fig. 3 A, compare lanes 4 and 5). As ERK1/2 has been shown to phosphorylate IE2-86 in vitro, the affect of U0126 on IE1-72 and IE2-86 phosphorylation was examined. Interestingly, phosphorylation of both IE proteins was significantly inhibited by U0126 (Fig. 3 B).
Fig. 3. Viral gene expression and IE protein phosphorylation in the presence of U0126. Serum-starved HEL fibroblasts were infected in the presence or absence of 10 µM U0126. At the indicated times, cells were harvested and analysed for viral protein expression by Western blot analysis. This was performed using antibodies to the immediate early proteins IE1-72 and IE2-86 (A, B), the early proteins UL44 and UL84 (C) and the late protein UL94 (D). For (B), infected fibroblasts were pulse-labelled for 2 h with [32P]orthophosphate (100 µCi/ml) prior to harvesting. After incubation, the cells were lysed, and IE1-72 or IE2-86 were immunoprecipitated from equal amounts of total protein, separated by electrophoresis, and subjected to autoradiography. Each experiment was performed at least three times, and a representative figure is shown. In each case, β-actin levels were determined by Western blot analysis to demonstrate equal protein concentrations between samples.

Early gene expression initiates at 8–24 h p.i., and is dependent upon both host cell transcription factors and IE proteins (Mocarski, 1996). In the presence of U0126, protein levels of two early gene products, UL44 and UL84 (Chambers et al., 1999; Chee et al., 1990; He et al., 1992), were decreased by 80–90% at 72 h p.i. (Fig. 3C, compare lanes 4 and 5). In addition, Northern blot analysis indicated that RNA levels of UL44, UL84 and two other early genes, UL54 and UL57, were decreased in the presence of U0126 (data not shown). Finally, protein levels of the true late gene UL94 (Chambers et al., 1999; Wing et al., 1996) were significantly lowered in the presence of U0126 (Fig. 3D, compare lanes 1 and 4 with 3 and 5). Since late gene expression does not begin until after viral DNA replication (Roizman, 1996), this result is consistent with the observation that inhibition of MKK1/2 kinase activity inhibits viral DNA replication. Thus, MKK1/2 kinase activity significantly affects early and late gene expression, and IE phosphorylation, but not IE gene expression.

MKK1/2 kinase activity may be essential for activation of one or more cellular transcription factors involved in HCMV early gene expression. Several transcription factors which mediate early gene expression have been identified, and we are currently examining how U0126 affects their transactivation potential following HCMV infection (Chau et al., 1999; Kerry et al., 1996, 1997; Rodems et al., 1998; Wade et al., 1992). While U0126 had a slight affect on IE gene expression at 8 h p.i., we believe this inhibition is not sufficient to alter the virus life-cycle or affect early gene expression, especially since the latter is still limited at 48–72 h p.i., when IE protein levels are not affected by U0126. U0126 does, however, have a strong affect on IE phosphorylation. Previous studies have shown that mutating ERK phosphorylation sites on IE2-86 actually increases its transactivation function in transient transfection assays (Harel & Alwine, 1998). However, it is possible that in the context of viral infection or different promoters, the ability of IE2-86 and/or IE1-72 to induce expression of early genes is enhanced by ERK1/2 phosphorylation. If so, this may explain in part the mechanism by which the ERK pathway alters early gene expression. Further work will need to be done to determine if this is indeed the case.

How does blocking MKK1/2 kinase activity inhibit viral DNA replication? Perhaps MKK1/2 kinase activity is necessary to activate one or more of the numerous cellular factors required for viral DNA replication. Alternatively, the protein products of UL44 and UL84, which were significantly decreased in the presence of U0126, are both required for viral DNA replication (Pari & Anders, 1993; Pari et al., 1993; Ripalti et al., 1995; Sarisky & Hayward, 1996). In addition, RNA levels of the early genes UL54 and UL57, whose protein products are also required for viral DNA replication, were decreased in the presence of U0126 (our unpublished data). Therefore, it is also possible that this overall decrease in the concentration of essential early proteins may be enough to inhibit initiation of viral DNA replication. Thus, HCMV may activate MKK1/2 and ERK1/2 to ensure sufficient expression of early genes, which in turn ensures timely initiation of viral DNA replication.

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