Trypsin increases pseudorabies virus production through activation of the ERK signalling pathway

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Extracellular proteases that are expressed in primary and secondary foci of viral infection are potentially important mediators of infectious inflammatory processes. For some viruses, such as influenza virus and rotaviruses, proteases such as trypsin enhance infectivity by a direct proteolytic effect on some virion proteins. By using an in vitro model of herpesvirus infection, the possibility that proteases modulate the viral cycle through signalling delivered to the infected cell was investigated. It is reported that exposure of pseudorabies virus-infected cells to trypsin increased virus production. Moreover, this treatment induced synergistic and sustained activation of the extracellular signal-regulated kinase (ERK) 1/2 signalling pathway, which appeared to be necessary for this increased viral production. These results suggest that herpesviruses could take advantage of the inflammatory context and particularly of the presence of proteases to increase their replication. Thus, these data point to a potentially important role of extracellular proteases in herpesvirus infection.

During viral infections, the sites of virus replication represent complex microenvironments whose effects on viral infection remain to be understood. In contrast to cytokines (Stark et al., 1998), the implication of proteases, which are produced in large amounts by inflammatory cells (Steinhoff et al., 2005), still remains enigmatic. Extracellular proteases are known to increase infectivity of several viruses through the cleavage of virion proteins necessary to pursue the viral life cycle (Zhirkov et al., 2002), but the possibility that such a phenomenon could be mediated through cell signalling has never been documented. The aim of our study was to test this hypothesis by using the herpesvirus model of Pseudorabies virus (PRV) (Klupp et al., 2004; Mettenleiter, 2002; Ray & Enquist, 2004). We report here that trypsin increases viral production by PRV-infected cells through activation of the ERK1/2 pathway.

In order to address the effect of trypsin on PRV replication, PK15 cells were infected (3 h, m.o.i. of 20) and then treated or not with trypsin (Becton Dickinson, ~ 410 BAEE units ml⁻¹, 50 μM, 5 min). We performed this delayed treatment to exclude the possibility of a direct effect of trypsin on virus particles. The cells were then returned to medium to let the infection proceed for 13 or 24 h. Viral growth was evaluated by titrating the virus in the supernatants using a plaque assay. When exposed to trypsin, PRV-infected cells subsequently produced more infectious virus (Fig. 1a, left panel), as indicated by the statistical Mann–Whitney test analysis (P < 0.05 at t = 24 h). Similar results were observed by using sucrose gradient-purified virus (Fig. 1a, right panel) and in Madin–Darby bovine kidney (MDBK; Fig. 1b) or ST (data not shown) cells. Thus, trypsin triggers an intracellular process leading to increased virus production in PRV-infected cells. To our knowledge, this is the first report demonstrating that proteases can mediate the enhancement of viral production through an effect on the infected cell. Whether this occurs through protease-activated receptors (Ossovskaya & Bunnett, 2004) and whether this is accompanied by an increased synthesis of virus proteins remain to be determined. Activation of the ERK1/2 intracellular signalling pathway is known to be associated with increased viral growth in several models of viral infection (Luo et al., 2002; Pleschka et al., 2001) and activation of this pathway has been reported in protease-mediated signalling (Brown et al., 2001; DeFea et al., 2000). Thus, in order to investigate its possible involvement in the increased virus production induced by trypsin in infected cells, PK15 cells were either mock- or PRV-infected and treated 3 h later with trypsin for 5, 30 or 120 min or left unexposed for the same length of time. Activation of the ERK1/2 pathway was then assessed by Western blot analysis using an anti-phospho-ERK antibody (Cell Signaling Technology). The results (Fig. 2a) showed that, in mock-infected cells, ERK1/2 was phosphorylated after 5 min exposure to trypsin, whilst after exposure for a longer time (between 30 and 120 min), the phosphorylation dropped below the basal level. These results were consistent with previous reports showing that trypsin treatment transiently activates the ERK1/2 pathway in several cell lines (Brown et al., 2001; DeFea et al., 2000). In contrast, virus and trypsin triggered a synergistic activation of ERK1/2 that...
Efficient signalling by Ras, an activator of the ERK pathway, implicates its translocation into lipid rafts (Dykstra et al., 2003; Roy et al., 1999). As virus may alter raft-dependent signalling (Avota et al., 2004; Favoreel et al., 2004; Mañes et al., 2003), we investigated the potential recruitment of ERK into these fractions. Raft analysis in PK15 cells, done as described previously (Riteau et al., 2003), showed that fractions 3, 4, 5, 10, 11 and 12 reacted with subunit B of the cholera toxin (Sigma-Aldrich) and anti-ERK1/2, respectively (Fig. 2d). These fractions were thus subsequently pooled and referred to as raft and soluble fractions, respectively. Rafts were then isolated from PK15 cells that were mock- or PRV-infected and later exposed or not to trypsin. Immunoblotting analysis using the cholera toxin and the anti-gE antibody showed that rafts were located similarly in all of the experiments and that they were selectively enriched in the mature form of the gE protein (Fig. 2e). Finally, whatever the cell treatment, most of the ERK1/2 proteins were detected in the detergent-soluble fractions (Fig. 2e), but only trypsin-treated infected cells displayed detectable raft-associated ERK1/2 that, moreover, appeared to be phosphorylated. Therefore, virus and trypsin induce the recruitment of ERK1/2 into rafts. As rafts act as signalling platforms (Dykstra et al., 2003) and are often hijacked by viruses to improve their replication (Mañes et al., 2003), rafts may be the starting point of the synergistic ERK1/2 activation. We finally tested whether a link could exist between ERK1/2 activation and the increased viral production in PRV-infected cells exposed to trypsin. PRV-infected cells were thus exposed or not to trypsin in the presence of either DMSO or 60 μM of the ERK1/2 signalling-pathway inhibitor U0126 (Promega), which worked efficiently under these conditions (Fig. 3a). The kinetics of virus growth were established for the four conditions tested by titrating infectious virus in the supernatant by a plaque assay (Fig. 3b). Trypsin treatment increased virus production by PRV-infected cells, but not in the presence of U0126, indicating that U0126 blocks the increased viral production induced by trypsin. In the absence of trypsin, U0126 also decreased PRV production, suggesting that, even in the absence of trypsin stimulation, ERK1/2 may be beneficial for virus replication. The pool of ERK responsible for this phenomenon could be the basal level of activated ERK observed in PK15 cells. Although we cannot exclude the possibility that the inhibition of PRV might be due to an intrinsic property of U0126, it is noteworthy that lactate dehydrogenase cell-viability assays (CytoTox96; Promega) showed that a 27 h treatment with U0126 had no toxic effect on PK15 cells. Altogether, these results suggest that the synergistic activation of the ERK1/2 signalling pathway observed in PRV-infected cells exposed to trypsin is increased over the time course of trypsin exposure in PRV-infected cells. The same results were also observed in MDBK (Fig. 2b, left panel) and RK13 (data not shown) cells and by using purified virus (Fig. 2b, middle panel). However, this was not observed with the heat-inactivated virus (Fig. 2b, right panel), in spite of its capacity to adsorb to the cells (data not shown), suggesting that productive infection is needed for the synergistic activation of the ERK1/2 pathway. Antibodies against ERK1/2 (Cell Signaling Technology) and PRV glycoprotein gE (Eloit et al., 1988) were used as controls. Similar results were also observed by using a lower concentration of trypsin, but only when PK15 cells were maintained in serum-free medium (Fig. 2c), suggesting that serum trypsin inhibitors block the phenomenon. Thus, PRV infection and subsequent trypsin treatment induce a synergistic activation of ERK1/2. This activation could be either direct or indirect through host factors released by infected cells. It would be interesting to investigate, for example, whether trypsin treatment results in activation of the epidermal growth factor receptor through release of transforming growth factor alpha (Darmoul et al., 2004).
necessary for increased viral production. Activation of this MAP kinase pathway seems to be specific, as the activation of JNK was not detectable in our experiments (data not shown). It is possible that ERK1/2 activation mediates increased viral production by affording cells an increased survival time, as described previously for several viruses (Luo et al., 2002; Perkins et al., 2003). Alternatively, ERK1/2 activation increases virus production directly by stimulating virus replication and/or expression of viral genes. Altogether, these studies suggest that proteases may play an important role in modulating virus pathogenesis.

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Fig. 3. Trypsin treatment increases viral-progeny production by PRV-infected PK15 cells in an ERK-dependent manner. (a) PK15 cells were grown, infected by PRV or mock-infected, treated by trypsin or left untreated, in the presence of either 60 μM U0126 or DMSO as in (b), except that trypsin treatment was for 50 min. At the end of trypsin treatment, aliquots of total cellular proteins were prepared and analysed by Western blot using the anti-phospho-ERK, anti-ERK and anti-gE mAbs. The numbers at the right of the figure refer to Mr in kDa. (b) Monolayers of PK15 cells were infected by PRV at an m.o.i. of 20. Three hours later, the cells were treated (●) or not (○) with 50 μM trypsin for 5 min and then replaced in normal medium. The experiments were performed in the continuous presence of either medium containing 60 μM U0126 (dotted lines) or DMSO (U0126 vehicle, solid lines) at the same dilution. After the indicated time post-infection, aliquots of culture supernatants were collected and infectious virus was titrated by plaque assay. The values given represent mean values ± SD from three independent experiments.

References


