Poliovirus (PV), the etiological agent of paralytic poliomyelitis, is the prototype member of the genus Enterovirus, of the family Picornaviridae (Pallansch & Roos, 2007). In a murine model, PV replication in the central nervous system (CNS) destroys motor neurons by triggering apoptosis (Girard et al., 1999). In neuronal cell lines, PV-induced apoptosis involves mitochondrial dysfunction (Belov et al., 2003; Gosselin et al., 2003). We have previously showed that this event was driven by a balance between pro- and anti-apoptotic signals: the JNK and PI3K pathways, respectively (Autret et al., 2007, 2008). The apoptotic programme initiated by PV infection also involves calcium (Ca\(^{2+}\)) flux from the endoplasmic reticulum (ER) to the cytosol during PV infection is involved in apoptosis induction in human neuroblastoma cells. We show here that PV infection is associated with a transient upregulation of Herp (homocysteine-induced ER protein), a protein known to promote the degradation of ER-resident Ca\(^{2+}\) channels. Herp gene transcription is controlled by the transcription factor CREB3 (cAMP response element-binding protein 3). We found that the CREB3/Herp pathway limited the increase in cytosolic Ca\(^{2+}\) concentration and apoptosis early in PV infection. This may reduce the extent of PV-induced damage to the CNS during poliomyelitis.
intramembrane proteolysis (RIP) pathway (Asada et al., 2011; Chan et al., 2011; Raggo et al., 2002). We had also identified CREB as interacting with the PV nonstructural protein 3A (Téoué et al., 2013), a multifunction protein involved in PV replication, both directly and indirectly, via massive remodeling of host intracellular membranes (Belov & van Kuppevelt, 2012; Paul & Wimmer, 2015; van Kuppevelt et al., 2010). We therefore hypothesized that PV-induced Ca\(^{2+}\) release from the ER and apoptosis might be modulated by the CREB3/Herp signalling pathway.

We tested this hypothesis by first investigating Herp up-regulation during PV infection in a neuroblastoma cell line (IMR5). IMR5 cells were inoculated with PV at an m.o.i. of 100 TCID\(_{50}\) per cell in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% heat-inactivated FBS (Gibco) (this m.o.i. was used for all assays performed in this study). Cells were also treated with thapsigargin (TG, 1 \(\mu\)M, Sigma-Aldrich T9033), a powerful Herp protein inducer (Kokame et al., 2000; Thastrup et al., 1990), as a positive control. Whole-cell extracts were collected at the times indicated and analysed by Western blotting as previously described (Téoué et al., 2013), with an anti-Herp antibody (19-Y, sc-100721, Santa Cruz) (Fig. 1a). Herp protein levels increased as early as 1 h post-infection (p.i.) and for up to 4 h p.i., corresponding to the early phase of PV replication in IMR5 cells (Brisac et al., 2010). Then, Herp protein levels decreased.

We then investigated whether the increase in Herp protein levels resulted from an activation of Herp transcription by monitoring Herp mRNA levels. Total RNAs were isolated from PV-infected IMR5 cells at the times indicated with the QIAGEN Total RNA Miniprep Kit (Sigma), and were reverse transcribed with the SuperScript II Reverse Transcriptase and Oligo (dT) (Invitrogen), according to the manufacturer’s instructions. The resulting cDNAs were subjected to quantitative real-time (RT)-PCR (Power SYBR Green PCR) Master Mix (Applied Biosystems) with specific primers (Table S1, available in the online Supplementary Material). Herp mRNA levels, determined by the 2\(^{-}\Delta\Delta C_{\text{t}}\) method (Livak & Schmittgen, 2001), began to increase early in infection, between 1 and 2 h p.i., reaching a plateau that was maintained until 6 h p.i., and decreasing thereafter (Fig. 1b). This pattern corresponds roughly to that observed for the Herp protein (Fig. 1a). PV infection thus induces a transient upregulation of Herp during the early steps of the virus replication cycle.

We then investigated whether the induction of Herp expression was dependent on CREB3 in our model using a short interfering RNA (siRNA) to downregulate endogenous CREB3 in IMR5 cells (siRNA-CREB3; ON-TARGET plus human CREB3 siRNA, L-017471, Dharmacon). Cells were reverse-transfected with the siRNA in the presence of Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. We checked the efficiency of CREB3 silencing by quantitative RT-PCR with specific primers (Table S1). The CREB3 mRNA signal was significantly weaker in IMR5 cells transfected with siRNA-CREB3 than in cells transfected with a non-targeting pool of control siRNAs (siRNA-ctrl; ON-target plus Non-targeting Pool, D-001810, Dharmacon) (Fig. 1c). We also checked that the siRNA-mediated downregulation of CREB3 had no effect on PV yield (data not shown).

IMR5 cells were then transfected with siRNA-CREB3 or siRNA-ctrl and infected with PV, or treated with the positive control TG. Total protein extracts were collected at the indicated time points and cellular Herp levels were analysed by Western blotting with an anti-Herp antibody (Fig. 1d). Herp levels were significantly lower in cells with endogenous CREB3 downregulation than in control cells.

These results indicate that PV infection induces a transient increase in Herp expression early in the infection cycle, and that this expression is CREB3-dependent.

We then compared the time courses of Herp expression (Fig. 1a) and cytosolic Ca\(^{2+}\) concentration during PV infection. IMR5 cells were incubated with the Ca\(^{2+}\)-sensitive dye Fluo3-AM [1,2-bis(o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid tetra (acetoxymethyl) ester, Life Technologies F-1242], and infected with PV. As a positive control, cells were treated with TG. At the indicated times p.i., cytosolic Ca\(^{2+}\) levels were determined by flow cytometry, as previously described (Brisac et al., 2010). As expected, PV infection resulted in an increase in cytosolic Ca\(^{2+}\) concentration, which was significant from 6 h p.i. onwards and reached a plateau at 16 h p.i. (Fig. 2a). Interestingly, cytosolic Ca\(^{2+}\) concentration increased with decreasing Herp levels (Fig. 1a).

We then assessed whether Herp accumulation at early time points in infection was involved in regulating the increase in cytosolic Ca\(^{2+}\) concentration in PV-infected cells. For this purpose, we selectively downregulated endogenous Herp levels with a specific siRNA (siRNA-Herp, Sigma; NM-001010989, SASI-Hs01-00063926). The efficacy of the siRNA-Herp in IMR5 cells was checked in cells treated with TG, by Western blotting with an anti-Herp antibody. As expected, Herp levels were significantly lower in cells transfected with siRNA-Herp than in cells transfected with siRNA-ctrl (Fig. 2b). Moreover, Herp downregulation did not affect PV yield (data not shown). Following infection with PV, cytosolic Ca\(^{2+}\) levels were higher in Herp-silenced cells than in siRNA-ctrl-transfected cells, from the 6 h p.i. time point onwards (Fig. 2c). This suggests that Herp induction early in PV infection limits the PV-induced increase in cytosolic Ca\(^{2+}\) concentration at later time points.

We then investigated the role of CREB3 in modulating the increase in cytosolic Ca\(^{2+}\) concentration, by downregulating endogenous CREB3 in IMR5 cells with a siRNA. Cells were transfected with siRNA-CREB3 or siRNA-ctrl, incubated with the Ca\(^{2+}\)-sensitive dye Fluo3-AM as described earlier, and infected with PV. Cytosolic Ca\(^{2+}\) concentration
Fig. 1. Transient upregulation of Herp expression during PV infection. (a) Herp protein levels increase early in PV infection. IMR5 cells were mock-infected or infected with PV and cell extracts were collected at the indicated time points p.i. Endogenous Herp was detected by Western blotting with an anti-Herp antibody. Actin was used as a protein-loading control. Cells treated for 8 h with TG (1 µM) were used as a positive control for the induction of Herp expression. Protein levels were determined by densitometry, with GeneTools image analysis software and expressed as a ratio relative to actin (arbitrary units). These values were also normalized against those for mock-infected cells (n-fold increase/mock). (b) Herp mRNA levels increase early in PV infection. IMR5 cells were mock-infected or infected with PV, and total mRNA was collected at the indicated times p.i. Herp mRNA was quantified by quantitative RT-PCR. The ß-actin housekeeping gene was used as a reference. *P<0.001 in Student’s t-tests comparing each set of conditions with mock-infected cells. (c) CREB3 silencing efficiency. IMR5 cells were transfected with 10 nM siRNA-ctrl or siRNA-CREB3. Total RNA was collected 36 h post-transfection, and CREB3 mRNA levels were determined by quantitative RT-PCR. The ß-actin housekeeping gene was used as a reference. The
was significantly higher in cells in which CREB3 was downregulated than in cells transfected with siRNA-ctrl, from 4 h p.i. onwards (Fig. 3a). These results suggest that CREB3 modulates cytosolic Ca$^{2+}$ concentration in PV-infected cells.

Finally, we assessed the effect of CREB3 on PV-induced apoptosis. The time course of PV-induced apoptosis was first analysed in IMR5 cells in the absence of silencing. IMR5-infected cells were stained with a metachromatic nuclear dye, the acridine orange (AO), to measure...
**Fig. 3.** CREB3 limits the increase in cytosolic Ca\(^{2+}\) concentration and the apoptosis driven by PV infection. (a) CREB3 silencing leads to an increase in cytosolic Ca\(^{2+}\) concentration in PV-infected cells. IMR5 cells were transfected with siRNA-ctrl or siRNA-CREB3. At 36 h post-transfection, cells were mock-infected or infected with PV, and cytosolic Ca\(^{2+}\) concentration, \([Ca^{2+}]_c\), was determined by flow cytometry with the Ca\(^{2+}\)-sensitive dye Fluo3 at the indicated times p.i. The increase (n-fold) in cytosolic Ca\(^{2+}\) concentration was calculated as the ratio of the percentage of fluorescent PV-infected IMR5 cells to the corresponding percentage of fluorescent mock-infected cells. (b) Apoptosis measurement (DNA fragmentation) in PV-infected cells. Mock-infected and PV-infected IMR5 cells were analysed at the indicated times p.i., by flow cytometry after staining with the nuclear dye acridine orange (AO). The percentages of apoptotic cells corresponding to a reduced fluorescence intensity (AO\(^{\text{low}}\)) are indicated. (c) CREB3 silencing leads to an increase in apoptosis levels in PV-infected cells. IMR5 cells were transfected with 10 nM siRNA-ctrl or siRNA-CREB3 and mock-infected or infected with PV, 36 h post-transfection. Cells were analysed at the indicated times p.i., by flow cytometry, after staining with the nuclear dye AO. The increase (n-fold) in apoptosis was calculated as the ratio of the percentage of apoptotic PV-infected IMR5 cells to the corresponding percentage of apoptotic mock-infected cells. The experiments were performed in triplicate and the error bars indicate the standard errors of the means. *P<0.05, **P<0.01 in Student’s t-tests comparing siRNA-ctrl-transfected IMR5 cells with siRNA-CREB3-transfected IMR5 cells.
chromatin condensation and fragmentation by flow cytometry, as previously described (Brisac et al., 2010; Estaquier et al., 1994). As expected, the time course of PV-induced apoptosis (Fig. 3b) paralleled the increase in cytosolic Ca\(^{2+}\) concentration in PV-infected cells (Fig. 2a). We then transfected IMR5 cells with siRNA-CREB3 or siRNA-ctrl and measured apoptosis at the indicated time points, following infection with PV. Consistent with the increase in cytosolic Ca\(^{2+}\) concentration upon CREB3 silencing (Fig. 3a), levels of PV-induced apoptosis were significantly higher in cells in which CREB3 was downregulated than in cells transfected with siRNA-ctrl (Fig. 3c).

Taken together, these results suggest that CREB3 limits the increase in cytosolic Ca\(^{2+}\) concentration and apoptosis in PV-infected cells, probably through Herp induction.

The induction of apoptosis during PV infection is controlled by a balance between survival pathways early in infection, allowing completion of the viral cycle, and the progression to programmed cell death later in infection (Agol et al., 2000; Autret et al., 2008; Belov et al., 2003). In this study, we showed that the CREB3/Herp signalling pathway was involved in modulating this balanced regulation. CREB3 is a transcription factor associated with ER stress and the ERAD pathway through activation of the Herp gene in particular (Asada et al., 2011; Chan et al., 2011; Liang et al., 2006). Herp plays a key role in ER stress recovery (Yan et al., 2014), and regulates Ca\(^{2+}\) homeostasis in the ER (Belal et al., 2012; Chan et al., 2004).

In PV-infected cells, we detected a transient activation of Herp gene transcription, resulting in increases in mRNA and protein levels. In particular, the amount of Herp protein increased until 4 h p.i., decreasing thereafter. The increase in Herp levels shortly after infection was dependent on CREB3, whereas the decrease in Herp levels probably resulted from a PV-induced shut-off of the transcription and translation machineries (Dougherty et al., 2010), given the short half-life of Herp (Sai et al., 2003).

The decrease in Herp levels followed the same dynamics as the previously described increase in cytosolic Ca\(^{2+}\) concentration during PV infection associated with cell progression to apoptosis (Brisac et al., 2010). We found that Herp restricted the PV-induced increase in cytosolic Ca\(^{2+}\) concentration. In addition, the downregulation of endogenous CREB3 led to an increase in cytosolic Ca\(^{2+}\) levels and apoptosis during PV infection. These results strongly suggest that CREB3 and Herp work as a signalling module, restricting PV-induced increases in cytosolic Ca\(^{2+}\) concentration and apoptosis.

As previously reported by our group (Autret et al., 2007, 2008; Brisac et al., 2010) and by Agol’s group (Agol et al., 2000; Belov et al., 2003), several series of events may be involved in the regulation of PV-induced apoptosis. The results presented here highlight a new pathway, the CREB/Herp pathway, which plays an anti-apoptotic role. This pathway transiently protects cells against PV-induced cell death and may provide the virus with sufficient time to replicate. We previously showed that the non-structural 3A protein of PV interacts with CREB3 (Téoulé et al., 2013). However, the role of the 3A protein in this process remains to be investigated.

CREB3 is produced in large amounts in the CNS (Ying et al., 2014), and Herp has been shown to regulate Ca\(^{2+}\) homeostasis in neurons and to prevent ER stress-induced apoptosis (Belal et al., 2012; Chan et al., 2004). These findings, together with our results, suggest that the CREB3/Herp module may be involved in the delicate balance between pro- and anti-apoptotic signals underlying the PV-induced neuropathogenesis of poliomyelitis.

Acknowledgements

We wish to thank Florence Colbère-Garapin for her invaluable support throughout this work and for fruitful discussions. We thank Santos Susin (Centre de Recherche des Cordeliers, Paris, France) and Victor Yuste (Autonomous University of Barcelona, Spain) for providing IMR5 cells. We also thank the Plate-forme de cytometrie (Pasteur Institut, Paris, France) for assistance with cytometry. This study was supported by grants from the Pasteur Institut (Transverse research programme PTR 276), the Agence Nationale de la Recherche (ANR-09-MIEN-019), and the Fondation pour la Recherche Médicale (DMP2009117313). C. M. was supported by a stipend from the Pasteur–Paris University (PPU) International PhD programme and by the Institut Carnot Pasteur Maladies Infectieuses. F. T. and C. B. were supported by grants from the Ministère de l’Enseignement Supérieur et de la Recherche.

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