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Role of Bcl-2 expression for productive herpes simplex virus 2 replication

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Abstract

Herpes simplex viruses infect a variety of cells in vitro. However, not all infected cells sustain a fully productive replication of these viruses. We have shown that, in U937 monocytoid cells, herpes simplex virus 2 (HSV-2) causes a low-productive infection characterized by apoptosis as cytopathic effect at a late stage of infection. This effect was associated with a down-regulation of the Bcl-2 protein. We therefore asked whether destabilization of Bcl-2 expression could act as a limiting factor for the productive HSV-2 infection. We found that overexpression of Bcl-2 in U937 cells dramatically increased the capability of these cells to sustain a fully productive infection, while protecting against apoptosis induced by HSV-2. Overall, our data indicate that Bcl-2 expression acts as a regulator of HSV-2 replication.

Keywords: Apoptosis; Bcl-2; Herpes simplex; Virus replication; Virus infection

Introduction

Although herpes simplex viruses (HSVs) infect a variety of human and nonhuman cell types, a post-entry restriction of infection occurs frequently. As a consequence, not all cell types potentially susceptible to HSVs can sustain fully productive replication of the viruses (Roizman and Knipe, 2001). Among these, lymphocytes, monocyte/macrophages and dendritic cells, despite harboring specific receptors that allow HSV attachment and entry, do not permit a full replication of the viruses. The significance of this phenomenon and the mechanisms controlling restriction of HSV infection in different, potentially susceptible cell types are poorly understood.

Full permissiveness to HSV infection is typically associated with cytolysis through the necrotic route, while abortive or low-productive HSV infection in immune cells is often associated with induction of apoptosis (Raftery et al., 1999; Fleck et al., 1999; Bosnjak et al., 2005). In fact, during evolution HSVs have selected redundant and apparently conflicting strategies to exert a fine control on apoptosis (Goodkin et al., 2004). This is not surprising as both herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) harbor in their genomes genes allowing them to cohabit with all the principle functions of the host cellular apparatus. Various viral and cellular genes, exerting a direct or indirect regulatory action on cell death during HSV infection have been identified. Particularly, studies from different groups proved that some HSV gene products play a role in preventing apoptosis. Concerning HSV-1, these include γ1 34.5 protein, infected cell protein 4 (ICP4), infected cell protein 27 (ICP27), Us3 protein kinase, glycoprotein J, glycoprotein D (gD) and a microRNA encoded by the latency-associated transcript gene (Chou and Roizman, 1992; Leopardi and Roizman, 1996; Aubert and Blaho, 1999; Munger et al., 2001; Hagglung et al., 2002; Zhou et al., 2000; Zhou and Roizman, 2001; Jerome et al., 2001; Medici et al., 2003; Gupta et al., 2006). Concerning HSV-2, the R1 protein kinase (ICP10 PK) has been shown to block apoptosis (Perkins et al., 2002). Regarding the cellular proteins which might potentially be

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involved as modulators of apoptotic cell death during HSV infection, members of the Bcl-2 family seem interesting candidates. In fact, the US3 protein kinase of HSV-1 has been shown to prevent apoptosis induced by different stimuli, through interaction with BH3-only pro-apoptotic proteins (Munger and Roizman, 2001; Cartier et al., 2003; Ogg et al., 2004). Moreover, overexpression of Bcl-2 blocked apoptosis induced by a mutant HSV-1 in a HEp-2 derived cell line and a retrovirus expression vector encoding *bcl-2* rendered HeLa cells resistant to the cytopathic effect of HSV-1 derived vectors (Galvan et al., 2000; Eling et al., 2000). In addition, wild type HSV-1 prevented p38MAPK-dependent destabilization of Bcl-2 in permissive cells (Zachos et al., 2001) and the HSV-2 R1 protein kinase increased the stabilization of Bcl-2 in cultured hippocampal neurons (Perkins et al., 2003). Finally, we demonstrated that the down-regulation of Bcl-2 was associated with induction of apoptosis by wild type HSV-2, as a late event occurring following restricted infection of U937 monocytoid cells Mastino et al., 1997). However, all these studies focused on HSV/Bcl-2 interaction in regulating apoptosis and not in controlling HSV replication.

We therefore asked whether destabilization of Bcl-2 expression could act as a major restriction factor for productive infection by HSV-2. Indeed, Bcl-2 overexpression in U937 cells allowed fully productive HSV-2 replication, while preventing apoptosis. Our results suggest that Bcl-2 expression could play a role in controlling the permissiveness/restriction state in HSV-2-infected cells.

Results

Restriction state in HSV-2-infected U937 cells is not due to a dysregulation in expression of the viral gene cascade

Infection of U937 cells by HSV-2 leads to the release of about 100 times less infectious particles than that released by fully permissive Vero cells (Mastino et al., 1997). We therefore asked whether this was due to an alteration in the expression of the HSV-2 gene cascade, during the replication cycle, in this cellular system. U937 cells were infected at a high multiplicity of infection (MOI) and, at various time points, total RNA was extracted from infected cells. The RNA was then reverse-transcribed and amplified using specific primers spanning over internal sequence of three representative genes for α, β and γ genes of HSV-2. RNA for the representative α gene product ICP22 was well detectable at 30 min after inoculum removal, i.e. at 90 min after first exposure to virus, after which it was practically stable (Fig. 1, upper panel). Maximal expression of RNA for ICP10/RR1 β gene product occurred at 3–5 h while that of the vhs γ gene product was further delayed (Fig. 1A). Contamination with genomic DNA was excluded by lack of PCR amplification products in the absence of reverse-transcription. Detection of RNA for vhs by reverse-transcriptase PCR as early as 90 min after exposure to virus, could be presumably ascribed also to the capacity of HSV to carry functional RNA in their viral particles, as demonstrated by some of us (Sciortino et al., 2002). A similar profile in the kinetics of

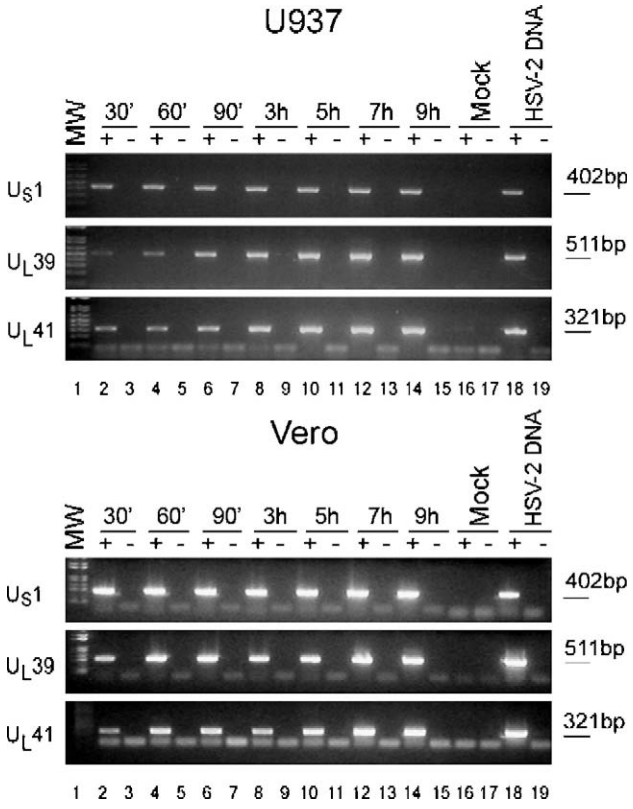


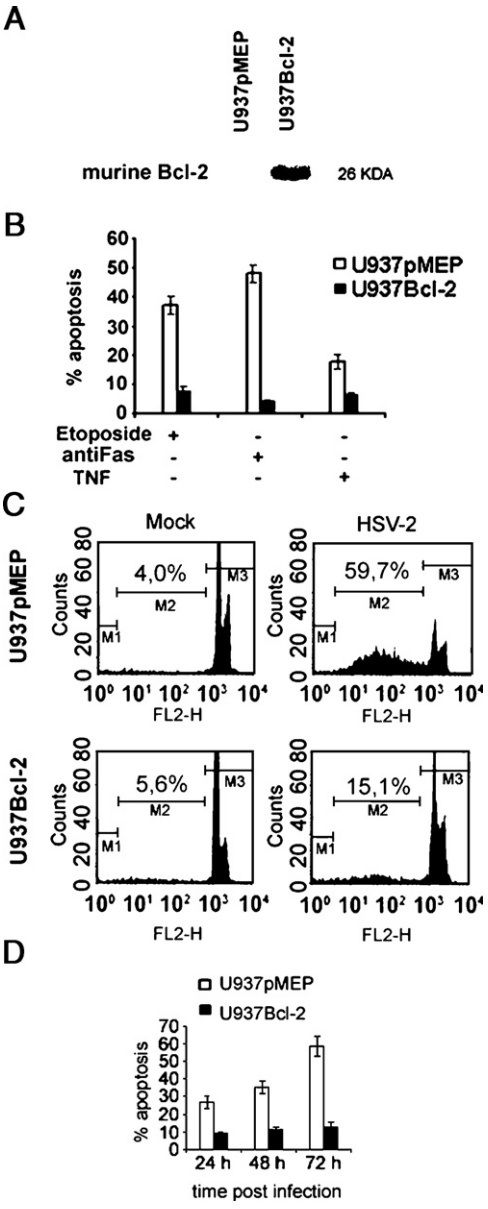
Fig. 1. Expression of viral gene cascade and in U937 and Vero cells infected by HSV-2. Cells were infected at a multiplicity of infection (MOI) of 50 PFU/cell (U937) or of 1 PFU/cell (Vero), respectively, with a "G" strain of HSV-2. At various times following infection, total RNA from infected cells was extracted, and 1.5 μg of RNA from U937 cells or 150 ng of RNA from Vero cells were reverse-transcribed and amplified using specific primers spanning over internal sequence of US1, UL39 and UL41 of HSV-2, respectively. PCR products were resolved on 2% agarose gel. Lanes 2, 4, 6, 8, 10, 12 and 14: reverse transcriptase was added to the reaction mixture. Lanes 3, 5, 7, 9, 11, 13 and 15: reverse transcriptase was omitted from the reaction mixture. Lanes 2 and 3: 30 min. Lanes 4 and 5: 60 min. Lanes 6 and 7: 90 min. Lanes 8 and 9: 3 h. Lanes 10 and 11: 5 h. Lanes 12 and 13: 7 h. Lanes 14 and 15: 9 h. Lane 16: PCR of DNA extracted from mock-infected cells, used as a template for positive control. Lane 17: PCR without DNA template as a negative control for mock-infected cells. Lane 18: PCR of DNA extracted from HSV-2-infected cells, used as a template for positive control. Lane 19: PCR without DNA template, as a negative control for HSV-2-infected cells. Lane 1: 1-kb plus DNA ladder. The positions of the amplified products are also shown.

expression of the same genes was observed in HSV-2 permissive Vero cells, infected at a low MOI (Fig. 1, lower panel) and HEp-2 cells (data not shown). From these experiments, we concluded that during the first phase of HSV-2 infection in U937 cells there was no block in the classical viral gene cascade and that the limited production of HSV-2 viral particles in these cells could be due to different events.

Bcl-2 overexpression protects U937 cells from HSV-2-induced apoptosis

We have shown that coincident with induction of MOI and time-dependent apoptosis, HSV-2 causes the down-modulation of Bcl-2 expression in U937 cells at a late stage of infection (Mastino et al., 1997). This suggests that the survival factor Bcl-

2 may play a role in regulating apoptosis caused by HSV-2. Given this possibility, we asked whether maintenance of high level Bcl-2 expression affects viability in U937 cells infected by HSV-2. We therefore used U937 cells stably overexpressing murine Bcl-2 (U937Bcl-2) (Borner, 1996) and compared them with cells carrying the expression vector alone (U937pMEP). Murine Bcl-2 expression was confirmed by Western blot analysis, using a mAb specific for murine Bcl-2 which does not cross-react with human Bcl-2 (Fig. 2A). To control functionality of overexpressed Bcl-2, we exposed U937pMEP and U937Bcl-2 cells to etoposide, an agonist anti-Fas antibody or high dose TNF. Overexpression of Bcl-2 rendered U937Bcl-2 cells highly resistant to all three apoptotic stimuli (Fig. 2B). Kinetic and MOI-dependency experiments proved that wild type U937 cells and U937pMEP cells were equally susceptible to HSV-2 infection, as detected by percentage of cells expressing virus-specific proteins using IFA or by virus yield titrated by plaque assay in Vero cells (data not shown). We then tested the capacity of U937Bcl-2 cells to resist apoptosis induced by HSV-2 at a high MOI. Flow cytometry analysis following propidium iodide staining at 72 h post infection demonstrated that while HSV-2-infected U937pMEP samples showed remarkable amounts of hypodiploid nuclei (Fig. 2C, upper right panel, M2), only a few such nuclei were detected in samples from HSV-2-infected U937Bcl-2 cells (Fig. 2C, lower right panel, M2). Similar results were obtained when apoptosis was detected by morphological analysis following staining with acridine orange, as shown in Fig. 2D that summarizes the time course of apoptosis up to 72 h, in HSV-2-infected U937pMEP and U937Bcl-2 cells (see also Supplementary material). Apoptosis of mock-infected U937pMEP as well as of U937Bcl-2 cells, in the same time course, was consistently low, showing a very narrow range of variation (minimum, 2.2%, maximum 5.3%). These experiments clearly show that Bcl-2 effectively protects U937 cells from HSV-2-induced apoptosis. Moreover, this protection persisted for the entire duration of the experiments, as HSV-2-infected U937Bcl-2 cells abruptly disintegrated starting from 6–7 days after infection, without evident signs of apoptosis (data not shown).



Bcl-2 overexpression overcomes the incapability of U937 cells to sustain a fully productive infection by HSV-2

We then wanted to know whether the death protection of U937 cells by Bcl-2 was associated with any changes in the

Fig. 2. Bcl-2 overexpression protects U937 cells from HSV-2-induced apoptosis. (A) Ectopic expression of the murine bcl-2 gene in U937 cells determined by Western blotting analysis. Proteins extracted from cells stably transfected with the expression vector alone (U937pMEP) or with murine Bcl-2 (U937Bcl-2) by lysis buffer were separated by SDS-PAGE electrophoresis, transferred to membrane, immunoblotted with a mAb specific for murine Bcl-2 which does not cross react with human Bcl-2 and visualized by ECL. The molecular weight is also shown. (B) Control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2) were treated with etoposide, an agonist anti-Fas antibody (antiFas) or tumor necrosis factor (TNF) and incubated for 18 h in 1% FCS medium. Apoptosis was then evaluated using fluorescence microscopy by calculating the percentage of cells showing the nuclear morphology of apoptosis after staining with acridine orange. Results are expressed as mean values±SD obtained from three replicate cultures. Comparison between corresponding groups using the Student's *t* test for independent samples was highly significant ($p<0.001$). (C) Apoptosis, evaluated using flow cytometry analysis of hypodiploid nuclei isolated and stained with a hypotonic solution containing detergent and propidium iodide, in control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2) mock-infected or infected with HSV-2 at a MOI of 50 PFU/cell. Apoptosis was detected 72 h after infection. The lines designated as M1, M2 and M3, indicate the boundaries among the peaks of diploid (M3), hypodiploid (M2) nuclei and presumably of debris (M1), which were arbitrarily set on untreated samples and maintained for all other samples. Percentages of hypodiploid (M2) nuclei are reported in the cytograms. (D) Apoptosis evaluated using fluorescence microscopy by calculating the percentage of cells showing the nuclear morphology of apoptosis after staining with acridine orange, in control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2) infected with HSV-2 at an MOI of 50 PFU/cell, at 24 h, 48 h and 72 h after infection. Results are expressed as mean values±SD obtained from three replicate cultures in one of the three experiments performed with similar results. Comparison of differences among the groups using the multiple comparison Bonferroni test was as follows: U937pMEP vs. U937Bcl-2, at all corresponding times, $p<0.001$; U937pMEP, 24 h vs. 48 h, NS; U937pMEP, 24 h vs. 72 h and 48 h vs. 72 h, $p<0.001$; U937Bcl-2, among all times, NS.

permissiveness of these cells to HSV-2. We first determined the infectivity of HSV-2 in U937Bcl-2 and U937pMEP cells by simultaneously staining the cells with a mAb specific for HSV-gD and with a mAb specific for endogenous human Bcl-2. No HSV-gD expression was detected, as expected, in mock-infected cells. In HSV-2-infected cells, flow cytometry analysis showed a higher expression of HSV-gD in U937Bcl-2 cells in comparison with U937pMEP control cells, particularly at 48 h after infection (Fig. 3, FL2-H). Moreover, control U937pMEP cells, unlike murine-Bcl-2 overexpressing cells, showed a decrease in the expression of endogenous Bcl-2 with the ongoing of the infection (Fig. 3, FL1-H). Interestingly, double fluorescence analysis clearly demonstrated a strict, direct correlation between endogenous Bcl-2 levels and the ability to support HSV-2 replication, as measured by gD protein expression, at single cell level. In fact, dot-plot analysis showed that both U937Bcl-2 and U937pMEP cells infected by HSV-2 were diagonally distributed in the cytograms, following Bcl-2/gD double-staining (Fig. 3, Bcl-2/gD). In addition, consistent with the above reported results, both Bcl-2 and gD were less expressed in U937pMEP than in U937Bcl-2 cells. Subsequently, we measured infectious virus from HSV-2-infected U937Bcl-2 cells and U937pMEP cells as assayed by plaque formation in permissive Vero cells. Fig. 4A shows the time course of virus yield following exposure to HSV-2 for 1 h. Both U937pMEP and U937Bcl-2 cells clearly showed a progressive increase in virus yield during the first 24 h. Moreover, at the same time point, the difference of virus titer between U937Bcl-2 and U937pMEP control cells was highly statistically significant, and this difference persisted also at 48 h and 72 h after infection (Fig. 4A). The continuously active replication of HSV-2 in U937Bcl-2 cells was confirmed by a semi-quantitative RT-PCR assay to detect the expression of the UL41 γ gene of HSV-2. In this assay, total RNA from infected or control cells was reverse-transcribed and cDNA was successively amplified using a reaction mix containing primer pairs specific for UL41 together with primer pairs specific for ribosomal 18S RNA, as a reference marker, before visualization of the PCR products on

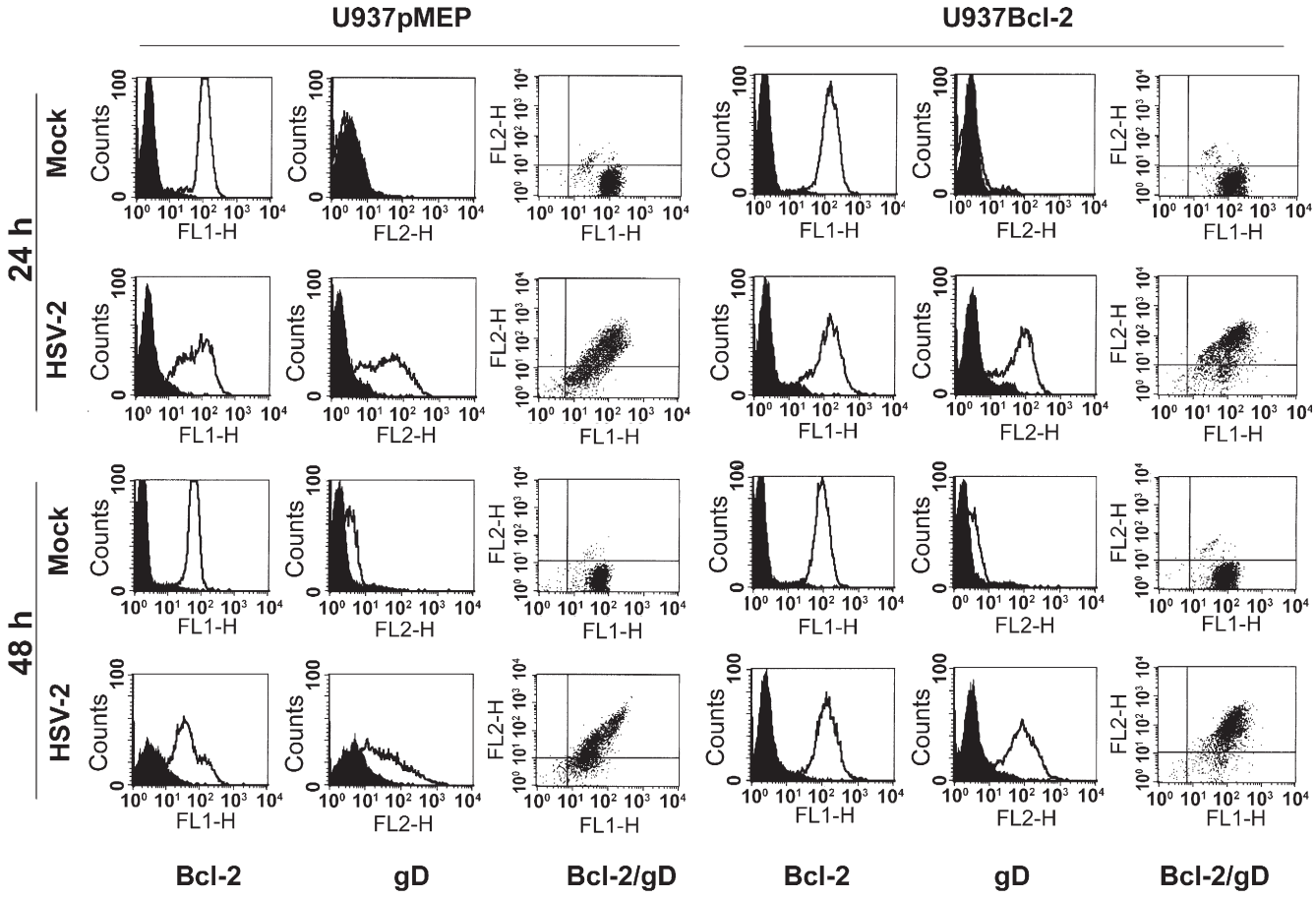


Fig. 3. Single- or two-color flow cytometry analysis of glycoprotein D and Bcl-2 in control and mBcl-2-overexpressing transfectants infected by HSV-2. Control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2) were mock-infected or infected with HSV-2 at a MOI of 50 PFU/cell. At 24 h and 48 h after infection, cells were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS with 0.1% FCS and incubated with a specific anti-gD mAb (FL2-H). Cells were then incubated with phycoerythrin-conjugated goat anti-mouse IgG, as a secondary antibody for gD detection, and successively with the fluorescein isothiocyanate-conjugated anti-human Bcl-2 mAb (FL1-H). Negative control staining was carried out by incubating control samples firstly with the phycoerythrin-conjugated secondary antibody only and, successively, with a fluorescein-conjugated mouse IgG isotype control antibody. Results are shown as single-color or two-color analyses of the same samples. Shaded histograms in single-color analysis represent control staining. Dashed lines in two-color analysis indicate the boundaries between negative and positive cells which were arbitrarily set for samples subjected to negative control staining (not shown) and maintained in all the corresponding Bcl-2/gD stained samples.

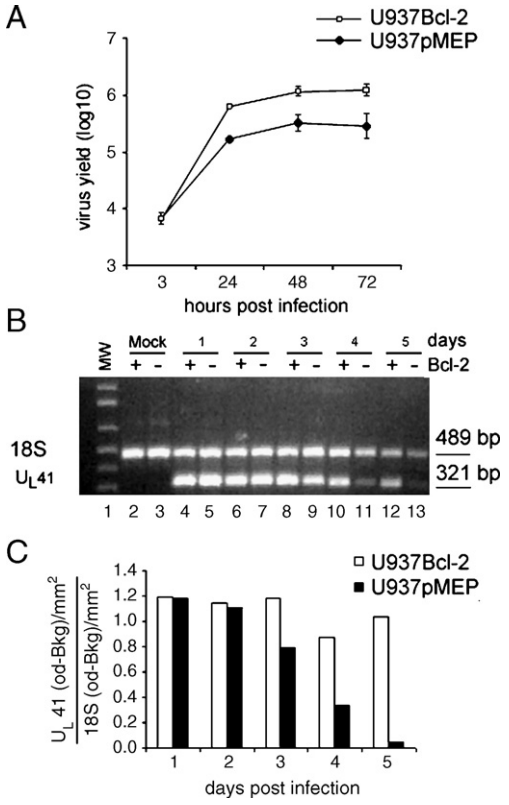


Fig. 4. Kinetic of virus yield and relative-quantitative PCR analysis in control and mBcl-2-overexpressing transfectants infected with HSV-2. (A) Total virus yield (lines) was titrated by plaque standard assay in Vero cells at 3 h, 24 h, 48 h and 72 h following infection of control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2) with HSV-2 at a MOI of 50 PFU/cell. Results are expressed as mean values ± SD obtained from three independent titrations. Comparison of differences among the groups using the multiple comparison Bonferroni test, was as follows: U937pMEP vs. U937Bcl-2, at 3 h, NS; U937pMEP vs. U937Bcl-2, at all other corresponding times, $p<0.001$; U937pMEP, 3 h vs. all other times, $p<0.001$; U937pMEP, among all times less 3 h, NS; U937Bcl-2, 3 h vs. all other times, $p<0.001$; U937Bcl-2, among all times less 3 h, NS; (B) relative-quantitative PCR analysis was carried out with the aid of the QuantumRNA 18S, control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2) were collected at different times after infection with HSV-2, and total RNA was extracted. Samples were then processed to eliminate possible viral DNA contamination and reverse-transcribed by random primers. The UL41 gene of HSV-2 and the 18S sequence, as an internal standard, were then amplified by PCR using specific primers. PCR products were resolved on 2% agarose gel. Lanes 2, 4, 6, 8, 10 and 12: U937Bcl-2 cells. Lanes 3, 5, 7, 9, 11 and 13: U937pMEP cells. Lanes 2 and 3: mock-infected. Lanes 4–13: cells infected with HSV-2. Lanes 1: 1-kb plus DNA ladder. The positions of the amplified products are also shown. (C) Densitometric analysis of PCR products shown in panel B, expressed as relative optical density following normalization with 18S.

agarose gel. As shown in Figs. 4B and C the UL41 gene transcripts remained highly detectable with increasing time of infection in U937Bcl-2 cells. Conversely, densitometric analysis demonstrated that UL41 transcript progressively decreased in U937pMEP starting from day 3 following infection (Fig. 4C). These results indicate that the pro-survival effect of Bcl-2 overexpression was associated with a remarkable prolongation of the capacity of U937 cells to sustain active HSV-2 replication. In contrast, in control U937pMEP cells, the production of infectious viral progeny was already impaired at 24 h after infection (Fig. 4A), obviously before appearance of massive HSV-2-induced apoptosis (Fig. 2C).

We also detected important changes in the pattern of HSV-2 replication between U937pMEP and U937Bcl-2 cells by electron microscopy analysis. In permissive cells, herpes simplex viruses replicate in the nucleus, where typical replication compartments are formed and profound nuclear changes occur (Roizman and Knipe, 2001; Monier et al., 2000). Both control U937pMEP cells and mBcl-2-overexpressing U937 cells showed a similar morphology at the beginning of infection (Figs. 5A and B, respectively). Particularly, no differences in mitochondrial structure (Figs. 6A and A1, respectively) or in morphology of viral attachment and entry (Figs. 6B and B1, respectively), were observed. However, at 24 h following infection U937pMEP cells started to exhibit morphological changes at both nuclear (chromatin margination, picnotic and deformed nuclei, Fig. 5C) and mitochondrial levels (Fig. 6C). Moreover, naked capsids with various morphologies, including well recognizable empty capsids, were detectable in the nuclei of these cells (Fig. 6D, arrow). At the same time after infection, also U937Bcl-2 cells showed chromatin margination, but they maintained cellular and nuclear integrity (Fig. 5D). Furthermore, most of the mBcl-2-overexpressing cells displayed large masses of naked capsids in nuclear compartments located in the inner margins of the nuclear membrane (Figs. 5D and 6D1, arrows). These formations had the typical features of paracrystalline,

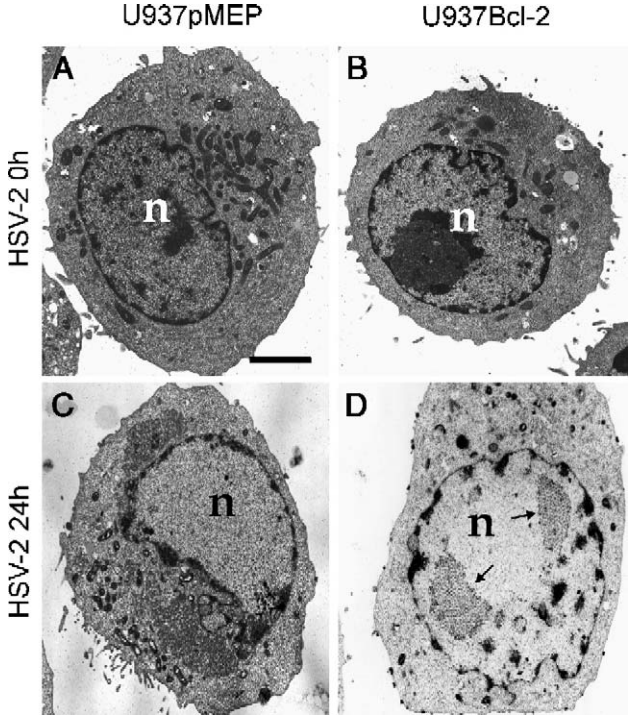


Fig. 5. Transmission electron microscopy of control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (U937Bcl-2). (A) Morphological feature of control U937pMEP cells at the beginning of infection. (B) Morphological feature of U937mBcl-2 cells at the beginning of infection. (C) Morphological feature of control U937pMEP cells 24 h after infection. (D) Morphological feature of U937Bcl-2 cells 24 h after infection. Arrows point to large masses of naked capsids inside the nucleus located beneath the inner side of the nuclear membrane. n: nucleus. Scale bar: 2 μm.

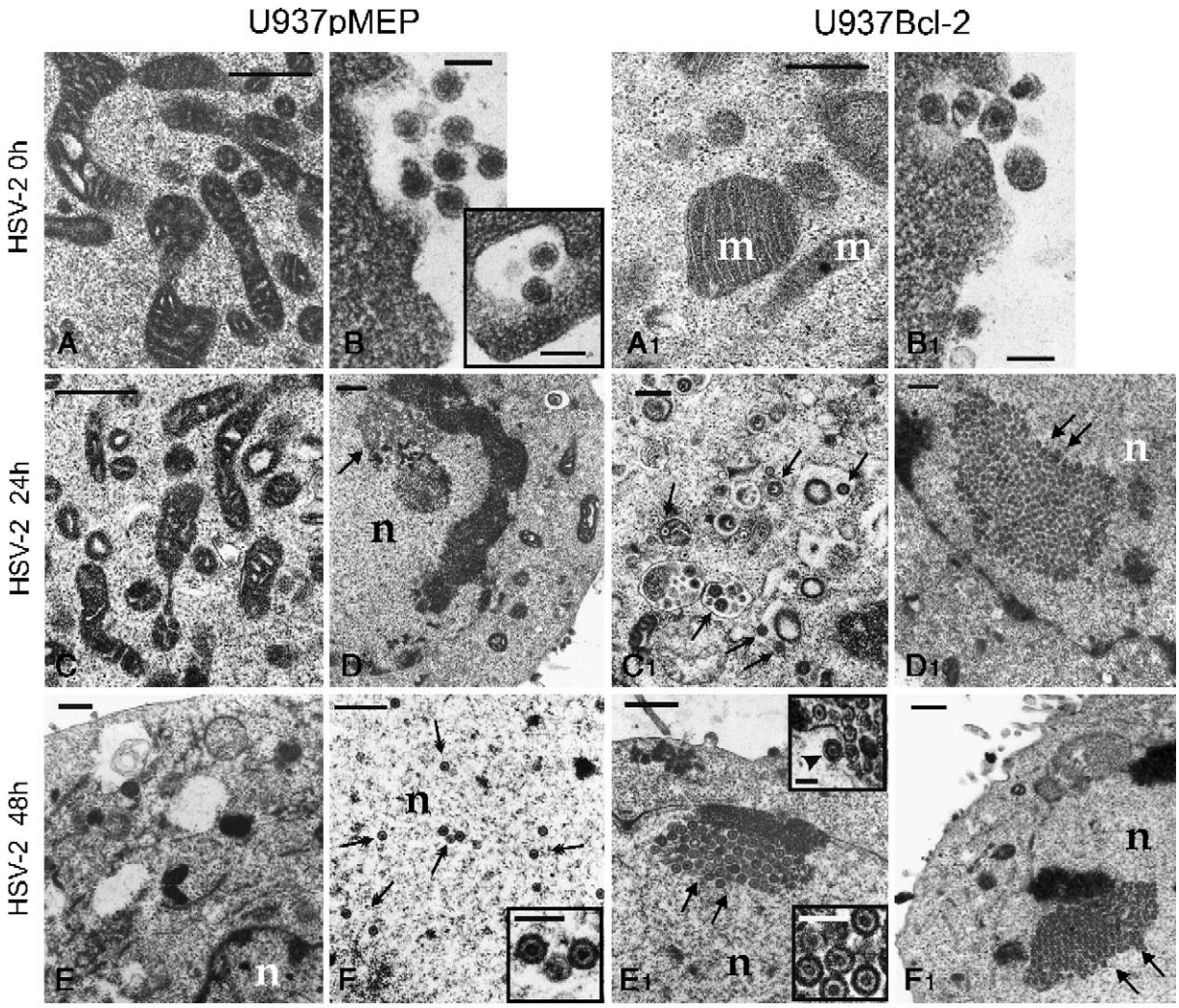


Fig. 6. Details of transmission electron microscopy of control transfectants (U937pMEP) or murine-Bcl-2-expressing transfectants (mBcl-2) infected with HSV-2. (A, B) Micrographs showing ultrastructural details of control U937pMEP cells at the beginning of infection. (A1, B1) Micrographs showing ultrastructural details of overexpressing U937Bcl-2 cells at the beginning of infection. (C, D) Micrographs showing ultrastructural details of control U937pMEP cells at 24 h after infection. (C1, D1) Micrographs showing ultrastructural details of overexpressing U937Bcl-2 cells at 24 h after infection. (E, F) Micrographs showing ultrastructural details of control U937pMEP cells at 48 h after infection. (E1, F1) Micrographs showing ultrastructural details of overexpressing U937Bcl-2 cells at 48 h after infection. Arrows point to naked capsids, clustered (D, D1, E1, F1) or scattered (F) inside the nucleus; double pointed arrows point to viral particles enveloped in cytoplasmic vesicles. Arrowhead in the upper inset in E1, points to enveloped capsid apparently budding at nuclear membrane level m: mitochondrion; n: nucleus. Scale bars: A, A1, C, C1, D, D1, E, E1, F and F1: 500 nm. B, B1 and details in the insets: 150 nm.

large regular aggregates of naked capsids described during productive HSV infection in permissive cells. Moreover, presumable tegumentation and envelopment of viral particles in the cytoplasmic compartments were observed frequently in U937Bcl-2 cells (Fig. 6C1) and very rarely in U937pMEP cells. At 48 h after infection, U937pMEP cells were remarkably damaged and showed clear signs of cytoplasmic disintegration (Fig. 6E). Single viral particles with various morphologies, all lacking an envelope, were still detectable scattered throughout the nucleus of some cells (Fig. 6F, arrows). Conversely, at this time, most of the U937mBcl-2 cells remained intact. They continued to show the typical morphology of HSV actively producing cells, with regular aggregates of naked capsids close to the inner leaflet of the nuclear membrane (Figs. 6E1 and F1, arrows; see also the insets). In summary, these results show that the morphology of U937Bcl-2 cells following HSV-2 infection remarkably resembled that classically described for HSV-infected fully permissive cells. Thus, prolonged overexpression of Bcl-2 apparently abrogated the post-transcriptional restriction of U937 cells to HSV-2 infection.

Inhibition of HSV-2-induced apoptosis by the pan-caspase inhibitor Z-VAD-FMK does not enhance virus production

We also wanted to verify whether inhibition of HSV-2-induced apoptosis by another mechanism could enhance virus production, similarly to what happened in murine-Bcl-2 over-expressing cells. To this purpose, we treated wild type U937 cells with the synthetic, cell-permeable, non-cleavable peptide analog Z-VAD-FMK to irreversibly inhibit protease activity

following infection with HSV-2 at a MOI of 50 PFU/cell. This peptide acts as a general caspase inhibitor. As shown in Fig. 7A, Z-VAD-FMK significantly reduced apoptosis of infected cultures, in comparison with control-treated cultures. This effect was particularly evident at 72 h after infection. However, Fig. 7B shows that treatment with Z-VAD-FMK, while almost completely blocked apoptosis induced by HSV-2 in U937 cells, did not substantially alter virus production as measured by plaque assay.

Discussion

Activation of the apoptotic pathway represents a first line, innate defense to restrict viral replication (Barber, 2001). On the other hand, control of the apoptotic machinery of infected cells, is one of the most powerful mechanisms for viruses to disarm non-specific resistance of the host (Benedict et al., 2002; Hay and Kannourakis, 2002).

In this context, several authors have focused their attention on Bcl-2, the founding member of a family of proteins playing pivotal roles in apoptosis (Borner, 2003). In fact, this anti-apoptotic protein could act as a key regulator in the balance between cellular apoptotic response and viral strategies of immune evasion, therefore largely influencing the final outcome of a viral infection. Related to this is the finding that various human herpesviruses encode viral homologues of Bcl-2 in their genomes (Hardwick and Bellows, 2003; Polster et al., 2004). Such an evolutionary convergence, based on Bcl-2 mimicry, suggests that maintenance of Bcl-2 functional activity seems to be an important strategy for the ineffectiveness of this family of viruses. However, Bcl-2 homologues have not been detected in the genome of HSVs, nor have any experimental result adequately explained the role of Bcl-2 expression in the infective cycle of these viruses. Nevertheless, observations reported by various groups, using different experimental models, including both HSV-1 and HSV-2, suggest a possible function of Bcl-2 in preventing apoptosis during HSV infection (Munger and Roizman, 2001; Cartier et al., 2003; Ogg et al., 2004; Galvan et al., 2000; Eling et al., 2000; Zachos et al., 2001; Perkins et al., 2003). Consistent with this hypothesis, we here show that overexpression of Bcl-2 is sufficient to completely interrupt the cascade of events leading to apoptosis in monocytoid cells infected with wild type HSV-2. However, the key new finding of our study was that U937 monocytoid cells, usually unable to efficiently sustain HSV-2 replication, became apparently fully permissive to the virus by means of Bcl-2 overexpression. Thus, based on our results, we propose that sustained Bcl-2 expression during HSV-2 infection not only serves a role in promoting cell survival, but also augments HSV replication, i.e. determines the permissiveness state of a cell for HSV infection.

While our results clearly outline the importance of proto-oncogene Bcl-2 expression in determining the host-cell permissiveness to HSV-2 infection, they do not provide a mechanistic explanation for the observed phenomenon. It remains to be determined exactly how Bcl-2 affects HSV replication in infected cells and how virus replication and apoptosis are mechanistically linked. One possibility is that a high level of Bcl-2 expression may ensure a productive infectious cycle by prolongation of the survival time of infected cells. Considering this assumption, a low level of Bcl-2 would be associated with premature apoptotic cell death and the inability to sustain HSV replication. If this is true, the decision point for survival or death exerted by Bcl-2 should be prior to virus replication. Bcl-2 is known to act at the mitochondrial level to inhibit the release of cytochrome *c* into the cytosol.

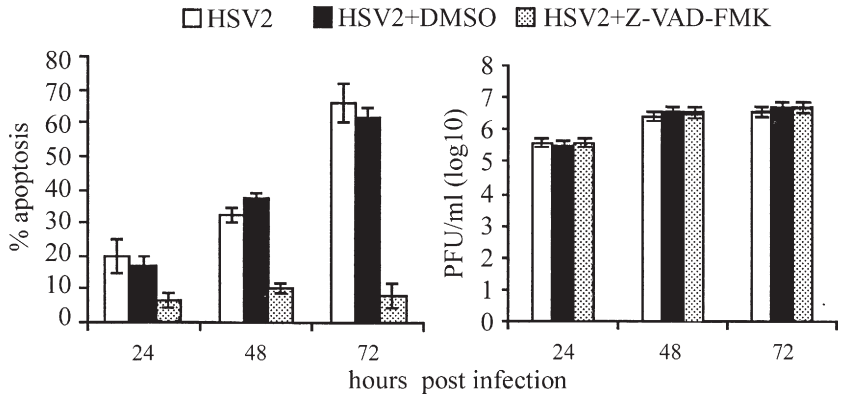


Fig. 7. Effect of caspase inhibition on apoptosis and on virus yield in U937 cells following HSV-2 infection. Z-VAD-FMK peptide analog of caspase substrates was added, at the concentration of 50 μM, to U937 cells infected with HSV-2 at a MOI of 50 PFU/cell, immediately after removing virus inoculum, and at half concentration daily. At different times after infection, apoptosis (left panel) and virus yield (right panel) were evaluated in HSV-2-infected untreated cells (HSV-2), in HSV-2-infected cells treated with DMSO control diluent (HSV-2+DMSO) and in HSV-2-infected cells treated with Z-VAD-FMK (HSV-2+Z-VAD-FMK). Apoptosis was evaluated using fluorescence microscopy by calculating the percentage of cells showing the nuclear morphology of apoptosis after staining with acridine orange. Total virus yield was titrated by plaque standard assay in Vero cells. Results are expressed as mean values±SD obtained from three replicate cultures. For apoptosis, comparison of differences among the groups using the multiple comparison Bonferroni test, was as follows: HSV-2 vs. HSV-2+DMSO, at all times, NS; HSV-2+Z-VAD-FMK vs. HSV-2+DMSO, at 24 h, NS; HSV-2+Z-VAD-FMK vs. HSV-2+DMSO at 48 h and at 72 h, $p<0.001$; HSV-2, 24 h vs. 48 h, $p=0.013$; HSV-2, 24 h vs. 72 h and 48 h vs. 72 h, $p<0.001$; HSV-2+DMSO, among all times, $p<0.001$; HSV-2+Z-VAD-FMF, among all times, NS. For virus yield, comparison of differences among the groups using the multiple comparison Bonferroni test, was as follows: among the three treatment-groups (HSV-2, HSV-2+DMSO, HSV-2+Z-VAD-FMK), at all times, NS; HSV-2, 24 h vs. 48 h and vs. 72 h, $p<0.001$; HSV-2, 48 h vs. 72 h, NS; HSV-2+DMSO, 24 h vs. 48 h and vs. 72 h, $p<0.001$; HSV-2+DMSO, 48 h vs. 72 h, NS; HSV-2+Z-VAD-FMK, 24 h vs. 48 h and vs. 72 h, $p<0.001$; HSV-2+Z-VAD-FMK, 48 h vs. 72 h, NS.

Cytochrome *c* release depends on the increased permeability of the outer mitochondrial membrane, an effect mediated by the pro-apoptotic Bcl-2 family members Bax and Bak (reviewed in Borner, 2003; Cory et al., 2003). While it is known that Bcl-2 can somehow inhibit the action of Bax and Bak (although the exact mechanism is not clear either), it is not yet understood how Bax and Bak are activated. A third subgroup of the Bcl-2 family, the BH3-only proteins serve as sensors for apoptotic stimuli which, by unknown mechanisms, convey intracellular apoptotic signals to the activation of Bax and Bak. It is therefore possible that HSV-2 activates one of these BH3-only sensor proteins. During the early phase of infection, high endogenous Bcl-2 levels would block BH3-only protein-dependent Bax/Bak activation and cells would be resistant to apoptosis. At later stages of infection, restricted cells, such as U937 cells, would undergo apoptosis when Bcl-2 is down-regulated. Conversely, virus replication would be sustained in permissive cells that maintain Bcl-2 levels. In any case, the question remains as to why Bcl-2 levels are down-regulated following HSV-2 infection in U937 cells, and not in permissive cells. Is this at the transcriptional or post-transcriptional level and is the down-regulation a cause or a downstream amplification effect of apoptosis? Bcl-2 might be cleaved and inactivated by caspases or other proteases activated during apoptosis. Interestingly, caspase-dependent Bcl-2 cleavage has been reported to potentiate apoptosis associated with Bcl-2 down-regulation (Liang et al., 2002). Particularly, inhibition of caspase activity during infection by apoptosis-inducing HSV-1 mutant viruses protected against decreased Bcl-2 levels and apoptosis, suggesting a caspase-dependent degradation of Bcl-2 protein during atypical, apoptosis-associated HSV infection (Zachos et al., 2001). The same study demonstrated that the down-regulation of Bcl-2 during infection by mutant viruses was mediated by two additional mechanisms consisting of decreases in bcl-2 RNA levels and p38MAPK-dependent decreases in the Bcl-2 protein half-life. Thus, at least in the experimental model of infection by apoptosis-inducing HSV-1 mutants, it seems that the decrease in Bcl-2 levels contributes to the amplification of virus-induced cell death, rather than causes apoptosis in the first place. If this is the case, another event rather than Bcl-2 down-regulation must play a role in switching the cell from survival to apoptosis during the course of HSV-2 infection. This event is most likely disabled in Bcl-2 overexpressing cells as these cells become resistant to HSV-2-induced apoptosis.

An alternative possibility is that the control of virus replication and cell survival by Bcl-2 are separate, even if interacting, events exerted by different molecular mechanisms. This is supported by the fact that the survival of fully permissive, naturally highly Bcl-2-expressing cells, which die by necrosis after HSV infection, is not longer than that of low-productive U937 cells that show Bcl-2 down-regulation and die by apoptosis following HSV infection. Moreover, our results on HSV-2 gene expression, viral yield and morphologic criteria by electron microscopy in semi-permissive U937 cells indicate that restriction in viral replication starts many hours before the appearance of apoptosis despite the fact that viral genome was fully expressed. Thus, prolongation of the survival time in infected cells does not seem to be a major discriminator between cells producing high or low viral yield. In addition, our results showing that inhibition of apoptosis by another mechanism, such as a pan-caspase inhibitor, did not lead to an increased virus production, indicate that prevention of apoptosis is not sufficient by itself to overcome the incapacity of U937 cells to fully sustain HSV-2 replication. Consequently, the augmented permissiveness to HSV-2 in Bcl-2 overexpressing cells is not simply related to the anti-apoptotic action of Bcl-2. Therefore, other mechanisms should be considered to understand the relationship between Bcl-2 expression and permissiveness to HSV-2 infection. Although the major function of Bcl-2 is to protect cells from apoptosis, other activities have been described. It has, for example, been demonstrated that bcl-2 gene expression can result in the inhibition of cell cycle progression at the G1 to S phase transition leading to a notable retardation of cell proliferation of several cell lines (Borner, 1996; Mazel et al., 1996). More recently, a new role for Bcl-2 as a mismatch repair activity suppressor has been identified (Youn et al., 2005). This latter function seems to be linked to Bcl-2-dependent cell cycle regulation, involving the hypophosphorylation of pRb and the enhancement of the E2F-pRb complex. All these findings indicate that Bcl-2 expression affects cell cycle progression. HSV infection is also associated with a block of the cell cycle and an arrest of host cell growth in the G1 phase (Song et al., 2000; Ehmann et al., 2000). This phenomenon involves expression of immediate-early viral proteins and interaction with cell cycle regulatory cellular proteins. However, the molecular mechanisms that control cell cycle progression during HSV infection are not well understood. In fact, they consist of complex, in some cases apparently contrasting interplay among viral and cellular factors that can change depending on multiple variables and cell types. For example, nuclear expression of cell cycle regulatory proteins cdk2 and cdk4 seems an essential requirement for HSV-1 productive reactivation in neurons (Schang et al., 2002) while productive infection of epithelial cells permissive to HSV-1 causes a block of cdk4 resulting in cell cycle arrest by decreasing pRb phosphorylation (Song et al., 2000). Thus, we can hypothesize that cell cycle regulatory activity exerted by high level Bcl-2 expression may be beneficial for productive infection by HSV-2 in our cellular systems, while down-regulation of the protein may be detrimental. Moreover, interestingly Bcl-2 localizes not only to mitochondria but also to the endoplasmic reticulum (ER), where it regulates the Ca^{2+} homeostasis (Foyouzi-Youssefi et al., 2000; Palmer et al., 2004), and to the nuclear envelope (Lithgow et al., 1994). In particular, it has been recently shown that phosphorylated Bcl-2 predominantly localizes to the ER (Bassik et al., 2004). Considering the central role of these cellular compartments for HSV envelopment and egress (Roizman and Knipe, 2001; Whiteley, 2001; Leuzinger et al., 2005), Bcl-2 control of HSV replication could occur also at this level. Finally, other molecular mechanisms may independently contribute to the low level virus production and apoptosis we observed in our study. Interestingly, it has been reported that HSV-1 fails to induce SOCS3 and IFN-dependent STAT1 phosphorylation in U937 cells (Yokota et al., 2004), suggesting

that low permissiveness of these cells to HSV infection and induction of apoptosis by HSV in the same cells could be related, at least in part, to an abnormally efficient IFN response to HSV.

In summary, we propose that Bcl-2 expression affects HSV-2 replication and hence acts as a major regulator of the permissiveness state of infected cells. On the other hand, presumably the virus accurately controls its replication and spread in different tissues by exerting a tight regulation of Bcl-2 stabilization/degradation. Further studies are necessary to confirm this hypothesis and to fully understand the role of Bcl-2 expression in HSV infection.

Materials and methods

Cells and virus

The sources and maintenance of U937 and Vero cells have been described elsewhere (Medici et al., 2003). U937 human monocytoid cells expressing the pMEP control vector (U937pMEP) or the vector containing the murine Bcl-2 (U937Bcl-2), produced for other purposes, have been described previously (Borner, 1996). They were maintained in culture as were wild type U937 cells, except for the addition of hygromycin B (50 μg/ml). Preliminary assays showed that these culture conditions were sufficient to warrant phenotypical and functional overexpression of the ectopic gene. Cells utilized in all experiments were derived from aliquots expanded at the initial passages, kept as frozen stock and used immediately after thawing and readapting to grow in culture. A "G" strain of HSV-2, originally provided by Prof. Bernard Roizman, University of Chicago, was used in these experiments. Virus stocks were produced and tittered on Vero cells. Infection of U937 cells was evaluated by means of a microscopy immuno-fluorescence assay (IFA) using a commercial HSV-2-specific monoclonal antibody (Kallestad, Sanofi Diagnostic Pasteur, Chaska, MN). The quantification of infectious viral particles was accomplished by a standard plaque assay. Serial dilutions of virus-containing cell-extracts were added to confluent monolayers of Vero cells plated in six-well plates and placed in constant agitation at 37 °C. After 1 h, the inoculum was replaced with complete medium containing 0.8% (wt/vol) methylcellulose (Sigma) and incubated for an additional 3 days. The plates were then stained with 1% (wt/vol) crystal violet in 20% methanol–80% H_2O (vol/vol), and the numbers of plaques were counted. Titers were calculated as PFU per milliliter of virus suspension. For experimental infections approximately 5×10^5 cells/ml were exposed for 1 h to HSV-2, as previously described (Medici et al., 2003), at a MOI of 50 PFU/cell. Preliminary experiments showed that this MOI corresponded to a virus inoculum able to productively infect about 50–60% of U937 cells, as evaluated by IFA at 24 h after infection.

Apoptosis assays

Infected and mock-infected U937pMEP and U937Bcl-2 cells were collected by centrifugation at different times following infection. The percentage of apoptotic cells was then evaluated using different techniques. Morphological analysis following staining with acridine orange and flow cytometry analysis of isolated nuclei stained with propidium iodide, carried out on a Becton Dickinson FACscalibur using CELLQuest II software, were performed as previously described (Mastino et al., 1997).

Antibodies and Western blot analysis

The anti-gD 1103/H170 mAb was kindly provided by Prof. Bernard Roizman. The anti-human-Bcl-2 fluorescein isothiocyanate-conjugated mAb, clone 124, was purchased from Dako. Western Blot analysis was carried out as previously described (Medici et al., 2003). After blocking, the blot was incubated overnight at 4 °C with a mAb to mouse/rat Bcl-2 (clone YTH 10C4, RD System) and incubated with a peroxidase-conjugated goat anti-mouse IgG (BioSurce). Antibody binding was detected by chemiluminescence staining using the ECL detection kit (Amersham Biosciences).

Flow cytometry analysis of gD and Bcl-2 proteins

For double-fluorescence gD/Bcl-2 detection, cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature and permeabilized in 0.1% Triton X-100 in PBS with 0.1% FCS for 5 min. After washing twice in PBS with 3% FCS, permeabilized cells were incubated with anti-gD mAb on ice for 30 min and washed twice in PBS. Cells were then incubated with a phycoerythrin-conjugated goat anti mouse IgG (Molecular Probes), as a secondary antibody for gD detection, for 30 min on ice and washed twice in PBS. Finally, cells were further incubated with the fluorescein isothiocyanate-conjugated anti-human Bcl-2 mAb for 30 min on ice and washed twice in PBS. Negative control staining consisted of incubation with the phycoerythrin-conjugated secondary antibody only, followed by incubation with a fluorescein-conjugated mouse IgG isotype control (R&D Systems). Stained cells were acquired and analyzed on a Becton Dickinson FaCScalibur using CELLQuest II software.

Reverse-transcriptase PCR and relative-quantitative PCR

For reverse-transcriptase PCR, an amount of 10^6 cells infected or mock-infected with HSV-2, were collected at different times post infection and lysed in TRIZOL (GIBCO BRL) to extract total RNA, according to the manufacturer's instructions. 1.5 μg of total RNA from U937 cells or 150 ng of total RNA from Vero cells, respectively, was reverse-transcribed with 60 U of AMV (Promega) in a total reaction volume of 30 μl. The primer sets used were: the forward primer, 5′-CGT-GTG-CGA-GCT-GCC-CTG-TCT-GAA-CGC-3′ and the reverse primer 5′-CGG-AGA-AAT-GTG-TGG-CCG-CAG-GGA-TAG-3′, located in the US1 gene of HSV-2; the forward primer, 5′-CCA-GTT-CAT-CGC-GCT-CAT-GCC-CAC-CGC-3′ and the reverse primer 5′-GAC-GAT-GTT-GTC-GTC-GCC-GGC-GAA-CAC-3′, located in the UL39 gene of HSV-2; the forward primer, 5′-GGA-ACA-CCG-

CGG-CTA-TGT-CGC-CGG-ACG-3′ and the reverse primer 5′-CAG-GAC-GTG-CTT-GTA-AAA-CGC-GGG-CGG -3′, located in the UL41 gene of HSV-2. Viral DNA of HSV-2 was extracted as described (Morse et al., 1977) and used as a template for positive control of the PCR products. The PCR was performed under the following conditions: 1 min 95 °C, 45 s at 60 °C and 2 min at 72 °C. PCR products were resolved on 2% agarose gel.

Relative-quantitative PCR utilized for the quantitative analysis of HSV-2 genes expression was performed using the QuantumRNA 18S internal standards kit, according to the manufacturer's instructions (Ambion).

Transmission electron microscopy (TEM)

For ultrastructural observation, U937pMEP and U937Bcl-2 cells were fixed with 2.5% glutaraldehyde in 0.1 M MPB containing 2% sucrose, and then post-fixed with 1% OsO4 in the same buffer. Cells were then dehydrated in ascending ethanol concentrations and embedded in Spurr epoxy resin (Agar Scientific Ltd.). Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Philips CM12 transmission electron microscope (Philips Instruments) operating at 80 kV.

Caspase-inhibition

For caspase-inhibition experiments, the pan-caspase inhibitor Z-VAD-FMK (Calbiochem), a cell-permeable peptide analog of caspase substrates, was added to infected and control cultures at the concentration of 50 μM, immediately after inoculum removal, and at half concentration daily. Apoptosis and total virus production were then assayed at different times by staining with acridine orange and plaque assay on Vero cells, respectively, as above described.

Statistical analysis

Comparisons of means were carried out using the Student's *t* test for independent samples or the Bonferroni multiple comparison test, where appropriate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.08.001.

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