Analysis of cyclins in trigeminal ganglia of calves infected with bovine herpesvirus-1

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Following acute infection of cattle with bovine herpesvirus-1 (BHV-1), cyclin expression was examined in trigeminal ganglia (TG). Cyclin A was primarily detected in the nucleus of TG neurons. In contrast, cyclin D1 and cyclin E were detected in the nucleus and cytoplasm of TG neurons. Uninfected or latently infected calves did not express detectable levels of these cyclins in TG neurons. Following dexamethasone-induced reactivation, cyclins D1, E and A were also detected in TG neurons. In situ hybridization of consecutive sections demonstrated that many neurons expressing cyclins contained viral nucleic acid, demonstrating that they were infected. Based on these observations, we hypothesize that BHV-1 infection activates neuronal cyclin expression to enhance productive infection. It is also possible that the stress of neuronal infection or reactivation leads to cyclin expression.

Bovine herpesvirus-1 (BHV-1) is a significant viral pathogen of cattle that can cause respiratory disease, abortions or occasionally encephalitis (Jones, 1998). Like other members of the Alphaherpesvirinae subfamily, BHV-1 establishes latent infection in sensory ganglionic neurons, primarily in trigeminal ganglia (TG). Viral DNA can persist in a latent state for the lifetime of an infected host but can periodically reactivate and spread to uninfected cattle. In contrast to the 70–80 viral genes expressed during lytic infection of bovine cells, viral gene transcription is severely impaired during a latent infection. The LR gene is the only abundant viral transcript in latently infected neurons (Hossain et al., 1995; Rock et al., 1987a, b; Schang & Jones, 1997). LR gene products arrest cells in the G1 phase of the cell cycle and an LR protein interacts with cyclin-dependent kinase 2 (cdk2)–cyclin complexes (Jiang et al., 1998; Schang et al., 1996). Expression of cell cycle regulatory proteins in neurons leads to apoptosis (Freeman et al., 1994; Gill & Windebank, 1998; Herrup & Busser, 1995; Shirvan et al., 1997a, 1998), suggesting that LR gene products enhance neuronal survival following infection. This hypothesis is supported by the finding that LR gene products inhibit apoptosis of transiently transfected cells (Ciacci-Zanella et al., 1999).

It is well established that small DNA viruses usurp cell cycle components to facilitate infection and there are several studies that suggest Alphaherpesvirinae members utilize cell cycle components for their infection cycle. For example, the herpes simplex virus type 1 (HSV-1) regulatory proteins ICP0 (Kawaguchi et al., 1997) and ICP22 (Bruni & Roizman, 1998) interact with cell cycle components. Mutations in the ICP0 (Cai & Schaffer, 1991) or VP16 gene (Daksis & Preston, 1992) are complemented by cellular factors that are expressed during G1 to S cell cycle progression. If HSV-1-encoded ICP0 cannot bind D cyclins, a virus expressing this mutant ICP0 is not neuroinvasive but can still replicate (Van Sant et al., 1999). Immediate early (IE) transcription is activated by cellular factors when growth arrest is released (Ralph et al., 1994) and cdk activity stimulates IE transcription and viral DNA replication (Schang et al., 1998, 1999). HSV-1 infection also induces S phase forms of a transcription factor (E2F) after infection (Hilton et al., 1995), alters nuclear localization of Rb (Wilcock & Lane, 1991), and rearranges nuclear structures that are sites for DNA replication (de Bruyn Kops & Knipe, 1988). Rb is a tumour suppressor protein that regulates cell cycle progression and is associated with E2F family members (Weinberg, 1995). Infection with HSV-2 induces transient activation of cdk2 and phosphorylation of Rb (Hossain et al., 1997). Finally, infection of cultured cells with pseudorabies virus stimulates cyclin E expression 1 h after infection (Hsiang et al., 1996). Although several studies have suggested that Alphaherpesvirinae members use cell cycle regulators for their infection, the issue is controversial and warrants further studies.

Cdk–cyclin complexes phosphorylate specific proteins resulting in cell cycle progression (Morgan, 1997). During G1 cell cycle progression, D-type cyclins (D1, D2 and D3) assemble into holoenzymes with cdk4 or cdk6. Late in the G1
phase cyclin E binds to cdk2 and it appears cdk2–cyclin E plays a role in initiation of DNA synthesis. Although overexpression of D- or E-type cyclins contracts G1, decreases cell size and reduce requirements for mitogenic stimuli, their functions are unique. Upon commitment to S phase, cyclin A and cdk2 complexes are detected. Cyclin A is associated with replicating DNA and is required for S phase entry and progression. During the G1 and M phase of the cell cycle cdc2–cyclin A and cdc2–cyclin B complexes are present. Although many of the substrates for cdk–cyclins have not been identified, Rb phosphorylation by cdk4–D cyclins and cdk2–cyclin A complexes occur during cell cycle progression.

To determine whether cyclins were induced in neurons of cattle after infection with BHV-1, we analysed thin sections prepared from TG at 7 days post-infection (p.i.), a time when extensive viral gene expression is detected in TG (Schang & Jones, 1997). The antibodies used for this study were initially tested for their specificity and lack of cross-reactivity with viral proteins. The respective antibodies specifically reacted with a single protein that migrated with the expected mobility (Fig. 1). Following infection of bovine kidney cells, additional bands were not detected, thus providing evidence that the antibodies did not cross-react with viral proteins. These studies also indicated that in actively growing bovine kidney cells the levels of cyclin D1, E and A did not change dramatically at 12 or 24 h.p.i.

Calves were infected with BHV-1 (Cooper strain) as described previously (Devireddy & Jones, 1998; Schang et al., 1996; Schang & Jones, 1997; Winkler et al., 1999, 2000) and cyclin expression was examined by immunohistochemistry (Schang et al., 1996; Winkler et al., 1999, 2000). Cyclin D1 protein expression was detected in the nucleus and cytoplasm of a subset of neurons (Fig. 2B) and satellite cells at 7 days p.i. (data not shown). Cyclin D1 was not detected in TG of two different uninfected calves (Fig. 2A and data not shown).

Intense cytoplasmic staining of TG neurons was observed when an antibody directed against cyclin E was used (Fig. 2C, D). Cyclin E protein expression was not detected in neurons or satellite cells of two different uninfected calves. At higher magnification (Fig. 2D), the nucleus of cyclin E-positive neurons frequently had a granular staining pattern and a novel organization. This unusual nuclear cytology was also evident in a subset of cyclin D1-positive neurons (Fig. 2B). Weak nuclear cyclin E staining was also detected in some neurons and a few satellite cells (Fig. 2D). Although cyclin E is regarded as a nuclear protein (Ohitsu et al., 1995), two reports have demonstrated that cyclin E can be localized to the cytoplasm. Anchorage-independent cells, transformed human fibroblasts and tumour cells, contain more than half of their cyclin E in the cytoplasm (Orend et al., 1998). In the same study, cyclin E was exclusively localized in the nucleus of normal human fibroblasts. Cytoplasmic localization of cyclins E and D1 also occurs in malignant human thyroid tissue (Lazzzereschi et al., 1998).

As previously described for the rabbit model of BHV-1 infection (Schang et al., 1996), cyclin A protein was detected in TG neurons using a monoclonal cyclin A antibody (Fig. 2E). In contrast to cyclin E, cyclin A expression was predominantly in the nucleus. Cyclin A was not detected in neurons of two different uninfected calves (Fig. 2F and data not shown). Cyclin B1 was not detected in TG neurons of calves infected for 7 days or mock-infected calves (data not shown). However, cyclin B1 was detected in a few non-neuronal cells, including mock-infected calves. After examining at least 1000 neurons stained with each antibody, we estimated that fewer than 10% of the total neurons were expressing cyclins after infection. The number of cyclin-positive neurons was lower than the number of neurons that contained viral nucleic acid. This may have resulted from the low sensitivity of immunohistochemistry or levels of cyclins may be decreasing because 7 days after infection is the transition from acute infection to latency.

We next examined whether cyclin expression occurred during reactivation and if cyclin-positive neurons were infected. A single intravenous injection of dexamethasone (DEX) reproducibly reactivates BHV-1 from latently infected calves (Winkler et al., 2000) and rabbits (Rock et al., 1992). Viral nucleic acids can be readily detected by in situ hybridization in TG neurons at 24 h after DEX treatment, but not prior to DEX treatment. At 24 h after DEX-induced reactivation, cyclins D1, E and A were detected in a small percentage of neurons (fewer...
Fig. 2. Cyclin expression in bovine TG at 7 days p.i. Thin sections were prepared from TG at 7 days p.i. and immunohistochemistry performed with the designated antibodies. The respective cyclin antibodies are described in Fig. 1. (A) Mock-infected TG incubated with the cyclin D1 antibody; magnification ×200. (B) Infected TG incubated with cyclin D1 antibody; magnification ×200. (C) Infected TG incubated with cyclin E antibody; magnification ×200. (D) Infected TG incubated with cyclin E antibody; magnification ×400. (E) Infected TG incubated with cyclin A antibody; magnification ×200. (F) Mock-infected TG incubated with cyclin A antibody; magnification ×200. Arrows point to neurons or a satellite cell (D) that were stained by the antibody.
Fig. 3. Detection of virus-infected neurons that express cyclins after DEX-induced reactivation. Latently infected calves (60 days p.i.) were treated with DEX to initiate reactivation (Winkler et al., 2000). TG were collected at 24 h after DEX treatment and consecutive sections processed for immunohistochemistry (IHC) or in situ hybridization (ISH). In situ hybridization was performed using RNase-free conditions as described previously (Winkler et al., 1999, 2000). Approximately 0.1 ng/ml of each probe was added to the prehybridization mixture. IHC was performed with the designated cyclin antibody. Magnification of cyclin D1 and E panels, × 250. Magnification of cyclin A panels, × 150.
than 5% of the total population) (Fig. 3). In general, the intensity of cyclin expression was lower at 24 h after DEX-induced reactivation compared to 7 days p.i. It was also noted that cyclin E was primarily localized to the nucleus of neurons after DEX treatment compared to 7 days p.i. We speculate that the stimulus for cyclin E expression is stronger or different during acute infection relative to reactivation and thus the difference in subcellular localization of cyclin E. It is also possible that at later times after reactivation cyclin E would be detected in the cytoplasm.

DNA probes specific for BHV-1 genes (ICP4, large subunit of ribonucleotide reductase, bICP0+LR gene, and gC) were synthesized and labelled by PCR as described previously (Hossain et al., 1995; Schang & Jones, 1997) using digoxigenin-dUTP (Boehringer Mannheim) in the reactions. The primers to amplify these viral genes and the conditions for PCR were described previously (Winkler et al., 1999, 2000). In situ hybridization of consecutive sections revealed that at least 50% of the cyclin-positive neurons contained viral nucleic acid. Prior to DEX treatment, we were unable to detect expression of cyclins (M. T. Winkler & C. Jones, unpublished data). In summary, these studies demonstrated that cyclin expression was stimulated in TG neurons after reactivation and viral nucleic acid was detected in some cyclin-positive neurons.

Expression of cell cycle regulatory proteins is frequently observed in neurons undergoing apoptosis (Freeman et al., 1994; Gill & Windebank, 1998; Herrup & Busser, 1995; Park et al., 1997a, b, 1998; Shirvan et al., 1997a, b, 1998). Furthermore, cell cycle regulators can initiate apoptosis (Levkau et al., 1998; Meikrantz et al., 1994; Meikrantz & Schlegel, 1996). Collectively, these observations suggested that virus-infected neurons expressing cyclins (acute infection or reactivation) are susceptible to death. The ability of LR gene products (Ciacci-Zanella et al., 1999) to inhibit apoptosis and interact with cdk–cyclin complexes (Jiang et al., 1998; Schang et al., 1996) may enhance survival of infected neurons. This hypothesis can now be tested directly because we have recently constructed a virus that has a mutated LR gene.

Although there seems to be compelling evidence that cell cycle components are important for BHV-1 replication and/or pathogenesis, it is possible that induction of cell cycle components has an antiviral effect. For example, overexpression of cdk2, cdk3 and cdc2 can induce apoptosis in neurons (Park et al., 1996) and cleavage of a cdk inhibitor is necessary for apoptosis (Levkau et al., 1998). Since BHV-1 induces apoptosis of cultured cells (Hanot et al., 1996, 1997, 1998; Devireddy & Jones, 1999) and in cattle (Winkler et al., 1999), induction of cell cycle components in the peripheral nervous system may lead to apoptosis and consequently prevent virus spread.

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