Short Communication

HveC (nectin-1) is expressed at high levels in sensory neurons, but not in motor neurons, of the rat peripheral nervous system

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The entry of herpes simplex virus (HSV)-1 into cells is a complex process mediated in part by the binding of the HSV glycoprotein D (gD) to a specific cellular receptor identified as HveC, or nectin-1. We examined the distribution of HveC in sensory and motor neurons of the peripheral nervous system (PNS) by immunocytochemistry. HveC is expressed at high levels in sensory neurons of dorsal root ganglion and their peripheral axons, at lower levels in motor neurons of spinal cord, and without detectable expression in motor nerve terminals at the neuromuscular junction. These results have implications regarding the tropism of HSV to specific neuronal populations, and for the construction of HSV-based vectors for the peripheral nervous system. *Journal of NeuroVirology* (2001) **7**, 476–480.

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The entry of herpes simplex virus into mammalian cells requires the participation of several viral envelope glycoproteins. The initial attachment of the virus to cell surface heparan sulfate proteoglycans is mediated by the glycoprotein C (gC) and/or gB (Herold *et al*, 1994; Herold *et al*, 1991). Viral penetration requires the fusion of the virion envelope with the cell plasma membrane, a process mediated by the viral glycoproteins gC, gB, and gH-gL acting in concert on cell surface receptors (Handler *et al*, 1996a; Handler *et al*, 1996b). Targeting of HSV to cells is achieved by several cell membrane proteins, originally identified as HSV entry (Hve) mediators. HveA, a member of the tumor necrosis factor receptor family, is the principal receptor responsible for entry of HSV into human lymphoid cells but not into other cell types (Montgomery *et al*, 1996). HveB, also known as the poliovirus receptor-related protein 2 (PRR2), mediates the entry of HSV-2, pseudorabies virus, and the HSV-1 mutants rid1, rid2, and ANG, but does not mediate the entry of wild-type HSV-1 into cells (Shukla *et al*, 1999). Neither HveA nor HveB fulfill the requirements for a coreceptor that can mediate the entry of HSV-1 into epithelial cells at the initial site of infection and into neurons for the establishment of latent infection.

The principal entry mediator of HSV-1 has been identified as HveC (Geraghty *et al*, 1998), a-518 amino acid type I membrane glycoprotein that is widely expressed in cells of epithelial and neuronal origin, and is nearly identical in sequence to nectin-1 (Shukla *et al*, 2000). The extracellular portion consists of three immunoglobulin-like domains (V-C2-C2, from the *N*-terminal to the membrane proximal domain) that contain seven potential glycosylation sites (Lopez *et al*, 1995). This family of proteins with similar structure, which in addition to HveB, HveC, and HveD include the poliovirus receptor (PVR or CD155), function in development as homophilic cell adhesion molecules. The intracellular carboxy terminal of HveC interacts with the PDZ domain of afadin, an f-actin binding protein, linking the extracellular action to the actin cytoskeleton (Takahashi *et al*, 1999). Because our experiments were carried out to investigate the role of this molecule in herpes entry, we have used the term HveC in this communication. HveC mRNA has been detected in a number of
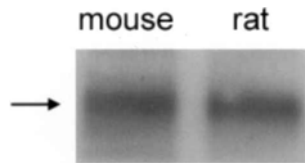

Figure 1 Western blot of rat brain protein. 20 μg of mouse (left) and rat (right) cortex was homogenized in lysis buffer, separated on a 4–15% SDS gel, transferred to a membrane, incubated with the anti-HveC antibody 1:1000 at 4°C overnight and then with a goat anti-rabbit IgG-HRP conjugate (1:10,000, BioRad) for 1 h. The immunoreactive band was detected by chemiluminescence (Super-Signal A, Pierce) with a 1-min exposure to X-ray film, and the resulting image digitized. Only a single major immunoreactive band was found, and was identical in the mouse and rat.

human cell lines including NT2 teratocarcinoma, SH-SY5Y, and the IMR-5 neuroblastoma, as well as primary cell cultures of fibroblast and skin keratinocytes (Geraghty *et al*, 1998). HveC is recognized by HSV gD independent of *N*-linked glycosylation, in a process that appears to involve one or two molecules of gD for each molecule of HveC. The sequences of gD involved in this interaction have been identified, and are conserved among all the

alpha herpesviruses (Krummenacher *et al*, 1998, 1999, 2000).

In wild-type infection, HSV is spread by epithelial contact, and progeny virions are taken up by sensory neurons innervating the area of the primary infection. In studies aimed at developing HSV-based vectors for therapeutic applications for the nervous system, we found that uptake of nonreplicating HSV recombinants into motor neurons from peripheral inoculation is substantially less than that into sensory neurons. This mimics the natural distribution of infection by wild-type virus. Although that distribution might result directly form the portal of entry, it prompted us to examine the distribution of HveC in the peripheral nervous system of mature rats.

The studies were performed with a polyclonal antibody (R154) raised against human HveC (Krummenacher *et al*, 2000) (a gift of Dr Gary Cohen) that has been previously characterized (Krummenacher *et al*, 1998, 2000). Mouse nectin has been cloned and is very similar to the human form (Shukla *et al*, 2000), but the rat isoform has not yet been cloned. Therefore, the specificity of antibody R154 for rat HveC was confirmed by comparing
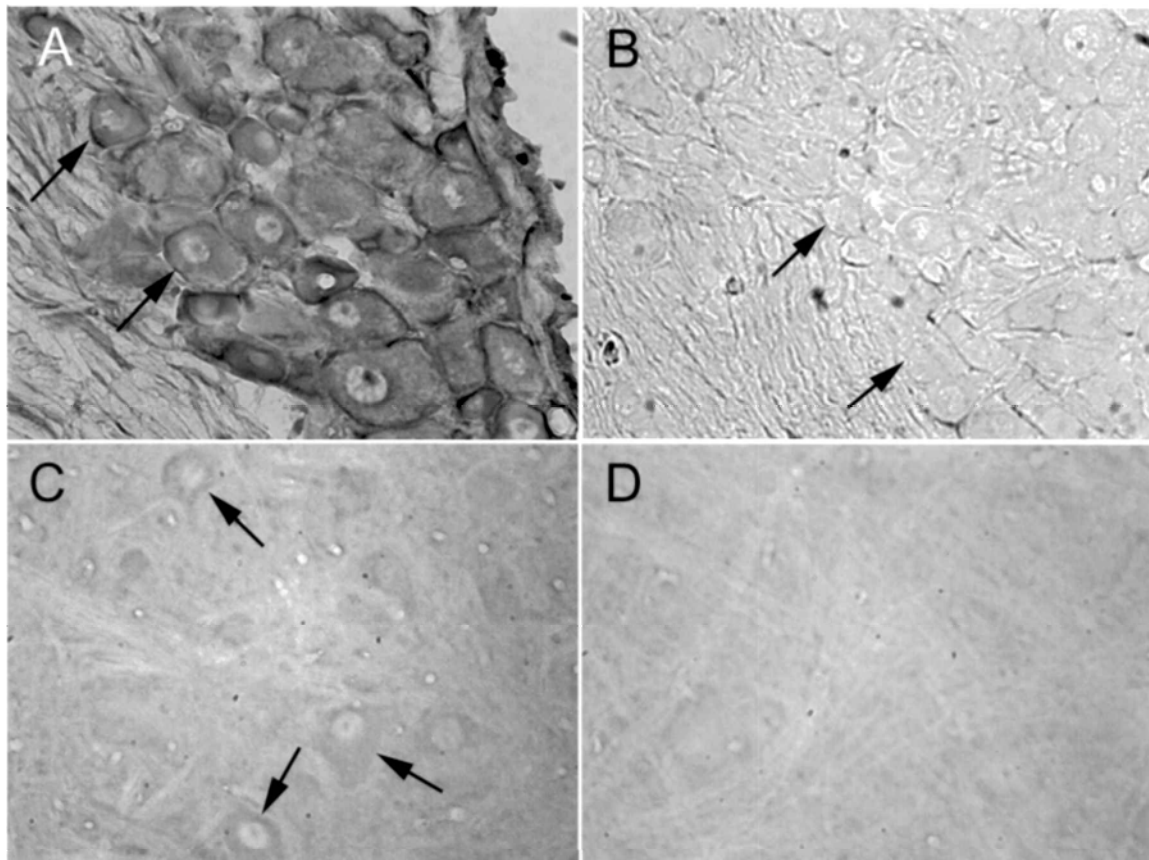
Figure 2 Immunostaining of DRG with anti-HveC demonstrates a high level of immunoreactivity in the sensory neuron cell bodies (black arrows, A). The nuclei are unstained. A control section, immunostained in a similar manner but with omission of the primary antibody shows no immunoreactivity in sensory neurons (black arrows, B). Immunostaining of ventral spinal cord with anti-HveC demonstrates only very faint immunoreactivity in motor neuron perikaryon (black arrows, C). A control spinal cord is shown in D.

Western blot of proteins from rat and mouse cortex, which demonstrated a single major immunoreactive band with an apparent MW of 67–70 kD, similar in size to the glycosylated human protein (Cocchi *et al*, 1998), that was identical in mouse and rat brain (Figure 1).

To examine the distribution of HveC in the rat peripheral nervous system, we performed an immunocytochemical study. Male Sprague–Dawley rats were perfused with saline followed by 10% formalin, and DRG and spinal cord removed, postfixed, and embedded in paraffin. Then, 4-μm sections were immunostained with the primary antibody (1:1000) followed by a biotinylated anti-rabbit IgG (1:200, Vector) and developed with diaminobenzidine (Vector Elite Kit, Vector laboratories). High levels of HveC immunoreactivity was found in neurons of the DRG (Figure 2), while less immunostaining was seen in motor neurons of the ventral horn of spinal cord (Figure 2).

The distribution HveC in sensory nerve terminals was examined in the footpads and digits. Fresh tissues were removed, fixed in 10% phosphate-buffered formalin at 4°C overnight, and postfixed in 30% sucrose at 4°C for 2 days. 30-μm sections were washed

with PBS blocked with 5% NHS in PBS-T for 30 minutes, then incubated with primary antibodies, anti-TuJ1 mouse monoclonal (1:250, Promega) and the anti-HveC rabbit polyclonal (1:1000), at 4°C overnight. After washing with PBS, the sections were incubated with anti-rabbit IgG conjugated to Cy2 (1:6000, Jackson Laboratories) and anti-mouse IgG conjugated to Cy3 (1:4000, Sigma) for 1 h at room temperature and following washing with PBS the mounted sections were examined with epifluorescence microscopy. HveC colocalized extensively with the axonal marker TUJ-1 in sensory nerves (Figure 3, left panels) and in the terminal branches in the subcutaneous tissue (Figure 3, A–D). HveC immunoreactivity alone is also seen in a distribution consistent with epithelial cells and fat cells that express relatively high levels of the protein.

To examine the distribution of HveC in motor nerve terminals, we studied the diaphragm, because the neuromuscular junctions are easily identifiable along the midportion of that muscle. The fresh diaphragm was removed, frozen in OCT at −90°C, and 10-μm sections washed with PBS, blocked with 5% NHS in PBS-T for 30 minutes, and then incubated with the
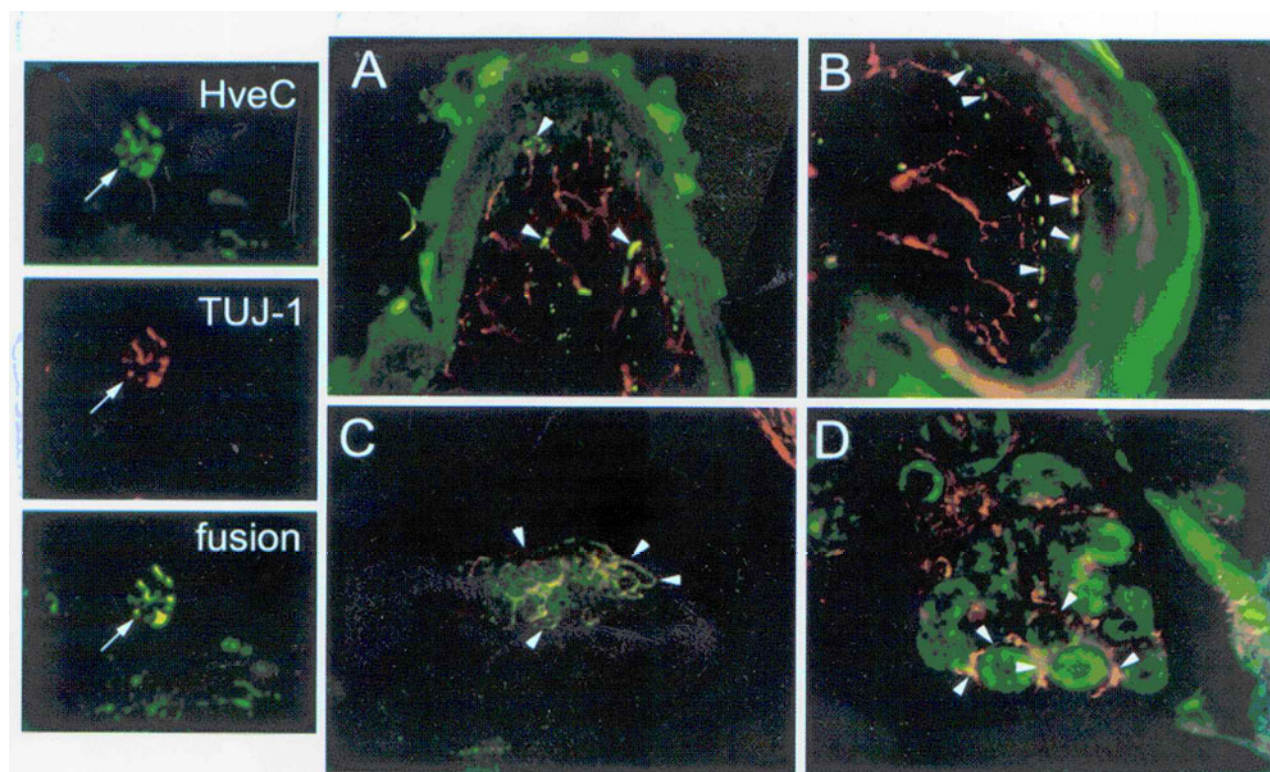
Figure 3 A sensory nerve branch in the foot, cut in cross section (white arrows) is shown immunostained for HveC, the axonal marker TUJ-1, and in a fused image. The extensive overlap of HveC and TUJ-1 in the nerve fibers is apparent. Sections of digits and footpad from the rat stained with antibodies against HveC and TUJ-1 (fusion image) shows extensive overlap of the two antigens (arrowheads, A–D). A and B show digits, cut in cross section; the extensive staining of epithelial cells with the HveC antibody outlines the digit, and the nerve fibers are seen within. C and D show footpads cut in cross section, with the dense plexus of nerve fibers characterized by the double immunostaining seen in this fused image. HveC immunoreactivity alone is also seen in a distribution consistent with epithelial cells and fat cells which express relatively high levels of the protein.
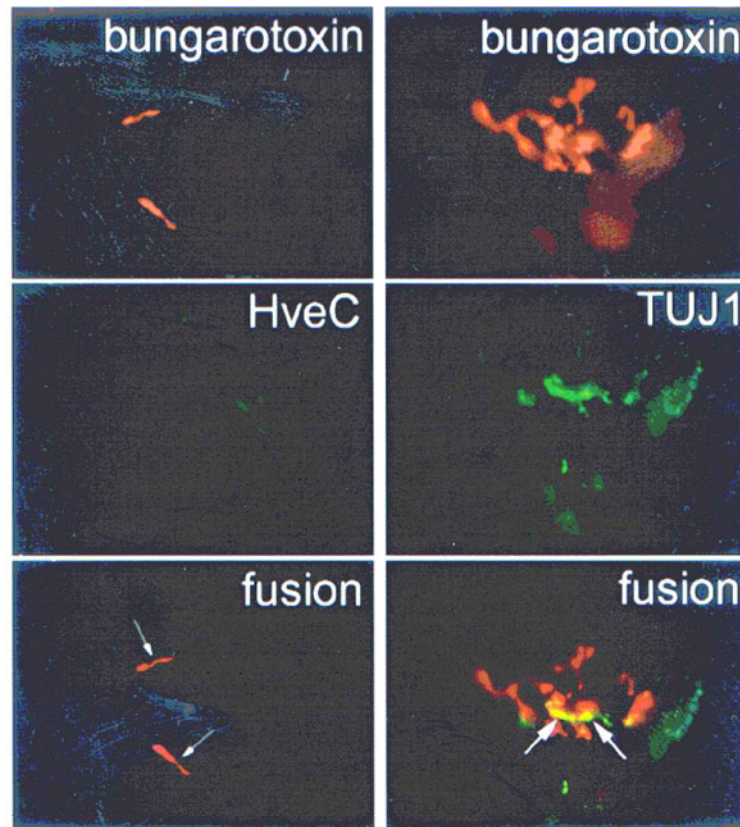
Figure 4 A motor endplate from the diaphragm, identified by bungarotoxin binding (top), shows no immunostaining with HveC (left panels). Motor nerve terminals present in this region can be identified with the antibody TUJ-1 (right panels) and overlap at their terminal portion with the motor endplate (fusion, right).

primary antibody (TUJ-1 mouse monoclonal or anti HveC rat polyclonal) at 4°C overnight. After washing with PBS the bound antibody was localized with the same anti-mouse IgG conjugated to Cy2 (1:4000, Jackson Laboratories) that had been used in the studies of skin. No immunostaining of nerve fibers was detected (Figure 4). To confirm that the terminal motor branches innervating the neuromuscular junction had been included, the sections were also incubated with fluorescently labeled α-bungarotoxin-TR (1:600, Molecular Probes) for 2 h at room temperature. As shown in Figure 4, the overlap of TUJ-1 with bungarotoxin binding confirms the localization of the motor nerve twigs, but no immunostaining against HveC is seen in these sections.

These studies demonstrate that the HSV entry mediator HveC is expressed to high levels in sensory neurons, where it is naturally transported from the terminal regions of axons of those cells. In contrast, motor neurons in spinal cord express low levels of HveC, and no detectable protein is found in the nerve terminals at the neuromuscular junction. Because HveC plays an important role in mediating the entry of HSV into cells, this data may shed some light on the tropism of HSV for sensory as opposed to motor neurons, and has important implications for the design of gene transfer vectors based on HSV.

Acknowledgements

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