Published in : Proceedings of the National Academy of Sciences of the United States of America (1996), vol. 93, iss. 5, pp. 2122-2125
Status : Postprint (Author's version)

Expression of protein encoded by varicella-zoster virus open reading frame 63 in latently infected human ganglionic neurons

Mahalingam R [1] , Wellish M [1], Cohrs R [1], Debrus S[3], Piette J [3], Rentier B[3], Gilden D H [1][2]

[1]Departments of Neurology, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Box B-182, Denver, CO 80262
[2]Departments of Microbiology, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Box B-182, Denver, CO 80262
[3]Laboratory of Fundamental Virology, Institute of Pathology, University of Liege, B-4000 Liege, Belgium

Abbreviations: VZV, varicella-zoster virus; ORF, open reading frame; HSV, herpes simplex virus; NRS, normal rabbit serum; IE, immediate-early.

Abstract

The ganglionic cell type in which varicella-zoster virus (VZV) is latent in humans was analyzed by using antibodies raised against *in vitro* -expressed VZV open reading frame 63 protein. VZV open reading frame 63 protein was detected exclusively in the cytoplasm of neurons of latently infected human trigeminal and thoracic ganglia. This is, to our knowledge, the first identification of a herpesvirus protein expressed during latency in the human nervous system.

Varicella-zoster virus (VZV) is an exclusively human herpesvirus that causes childhood chickenpox (varicella), becomes latent in dorsal root ganglia, and frequently reactivates decades later to produce shingles (zoster), mostly in the elderly. Unlike latent herpes simplex virus (HSV), VZV cannot be rescued by explantation or cocultivation of ganglionic cells with indicator cells *in vitro*. Nevertheless, postmortem analysis of latently infected sensory ganglia at all levels of the neuraxis revealed low levels of virus DNA (1) from multiple regions of the VZV genome in most humans (2, 3), including transcripts from no less than three regions of the VZV genome (4-6). Analysis of latently infected ganglia by *in situ* hybridization has identified VZV nucleic acid in neurons (7, 8), including ganglionic neurons in an animal model of VZV latency (9, 10), and in satellite cells (4, 11). The latter finding was surprising, since VZV DNA is not integrated into cell DNA (12). Thus, the biology of VZV latency and reactivation is best explained by virus residing in and reactivating from nondividing neurons. Since antibodies against *in vitro* expressed product of VZV open reading frame (ORF) *63* were successfully used to detect viral antigen in rat ganglia (13), we used the same antibodies to analyze the ganglionic cell type in which VZV is latent in humans.

Methods

Tissue Samples

One trigeminal and multiple thoracic ganglia from nine adults and three infants were obtained within 24 hr after death. None of the subjects was immunocompromised before death, and at autopsy there were no cutaneous signs of recent herpesvirus infection. The tissue samples were para-formaldehyde-fixed and paraffin-embedded. Sections (5 μm) were deparaffinized with 100% xylene for 5 min followed by 100% ethanol for 5 min. The xylene-alcohol treatment was repeated twice.

Immunohistochemistry

Unless indicated otherwise, all incubations were at room temperature. The deparaffinized sections were

Published in : Proceedings of the National Academy of Sciences of the United States of America (1996), vol. 93, iss. 5, pp. 2122-2125
Status : Postprint (Author's version)

incubated with a 10% (vol/vol) solution of normal sheep serum for 1 hr followed by a 1:100 dilution of either rabbit antiserum directed against the VZV ORF 63 protein (13) or normal rabbit serum (NRS) in phosphate-buffered saline (PBS) for 30 min. Both rabbit sera had been preabsorbed with normal human liver powder for 30 min and again for 20 hr at 4°C. Sections were rinsed with PBS, incubated for 20 min with a 1:300 dilution of biotinylated goat anti-rabbit IgG (DAKO) in PBS containing 5% NSS, washed three times with PBS, incubated for 20 min with a 1:100 dilution of alkaline phosphatase-conjugated streptavidin (DAKO), and washed three times with PBS. The color reaction was developed for 5-30 min with fresh fuchsin substrate system (model K0698; DAKO). Levamisole was added to the color reaction at a final concentration of 24 μg/ml. Sections were counterstained with Gill's hematoxylin for 4 min, rinsed in water and mounted by using an aqueous medium. Except for the deparaffinization step, VZV-infected and uninfected BSC-1 cells grown on coverslips and fixed in acetone were treated identically.

Results and discussion

To analyze VZV expression in latently infected ganglia, one trigeminal and multiple thoracic ganglia from nine adults and three infants were obtained within 24 hr after death. Enzyme immunoassay (14) revealed antibody to VZV in the serum of all of the adults. Serum available from only one of the three infants did not contain antibody to VZV. Liquid PCR amplification with VZV-specific primers (3) revealed VZV DNA in all adult ganglia but not in any infant ganglia in DNA extracted from a small portion of each ganglion (data not shown). The remaining portions of each ganglion were analyzed immuno-histochemically by using antibodies raised in rabbit against the 30.5-kDa-VZV ORF 63 protein, expressed as a glutathione S-transferase fusion protein in *Escherichia coli* and purified as described (13). VZV-infected and uninfected BSC-1 (African green monkey kidney) cells served as positive and negative controls, respectively.
In VZV-infected BSC-1 cells, two focal areas of typical VZV cytopathology containing VZV ORF 63 protein were seen (Fig. *A).* No signal was detected in uninfected cells (Fig. 1*B)* or in HSV-infected BSC-1 cells (not shown) or when NRS was applied to VZV-infected BSC-1 cells (not shown). In ganglia acquired 17 hr after death from a 46-year-old man who died of atherosclerosis, a characteristic red color revealed VZV ORF 63 protein exclusively in the cytoplasm of three neurons (Fig. 1C). No signal was detected when NRS was applied to an adjacent section of the same ganglion (Fig. 1D). A nonspecific brown color in neurons (Fig. 1 *C* and *D)* represents pigmentary changes often seen in hematoxylin-stained human ganglia. Intense red staining was detected in the cytoplasm of a neuron in adjacent sections of a different dorsal root ganglion of the same subject (Fig. 1*E* and *F).*

Published in : Proceedings of the National Academy of Sciences of the United States of America (1996), vol. 93, iss. 5, pp. 2122-2125
Status : Postprint (Author's version)

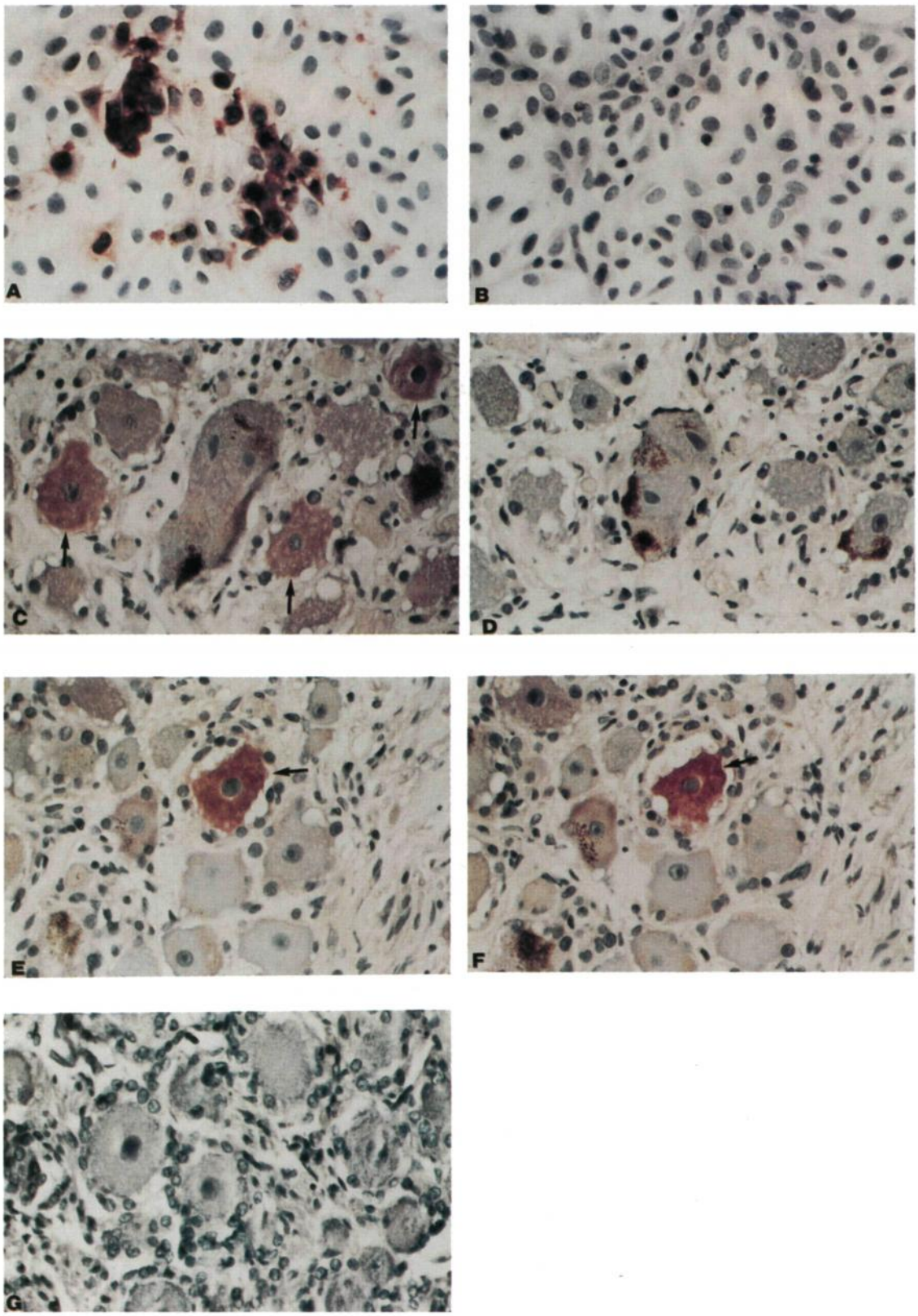

FIG. 1. Detection of VZV ORF 63 protein in human thoracic ganglia latently infected with VZV. (A and B) VZV-infected (A) and uninfected BSC-1 cells (B) incubated with antiserum raised against VZV ORF 63 protein (anti-63). (x 77.) (C and D) Section of a thoracic ganglion obtained from a VZV-seropositive 46-year-old man and incubated with anti-63 (C) or NRS (D): arrows point to three neurons positive for VZV ORF 63 protein. (x122.) (E and F) Adjacent sections of a different thoracic ganglion from the same subject and incubated with anti-63: arrows point to neurons positive for VZV ORF 63 protein. (x122.) (G) Thoracic ganglion derived from a VZV-seronegative 2-month-old infant

and incubated with anti-63. (x122.)

A lighter red color was also often seen in multiple neurons of latently infected ganglia, suggesting a more diffuse, perhaps lower abundance infection. The red VZV ORF 63 protein-specific staining was never seen in satellite (capsular) cells, capillaries, connective tissue, nerve fascicles within the ganglia, or nerve rootlets entering the ganglia. No signal was detected when the antiserum against VZV ORF 63 protein was applied to ganglia of a 2-month-old infant who died of sepsis complicating a congenital omphalocele and pulmonary hypoplasia (Fig. 1G).

Overall, VZV ORF 63 protein was found in five ganglia (four thoracic and one trigeminal) from two adults. In one of these adults, VZV ORF 63 protein was detected in all 10 sections from four of five thoracic ganglia, but not in any of 10 sections from the fifth thoracic ganglion. In the second adult, VZV ORF 63 protein was detected in all four sections from the trigeminal ganglion. In positive sections, VZV ORF 63 protein was detected in the cytoplasm of two to four neurons. VZV ORF 63 protein was not detected in any of four sections prepared from each of 16 ganglia from seven other adults, or in any of four sections from each of nine ganglia from three infants. The detection of VZV DNA in nearly all ganglia but VZV ORF 63 protein only in some latently infected ganglia might reflect sampling—i.e., analysis of every section of each ganglion containing VZV DNA for VZV ORF 63 protein might have revealed additional positive ganglia. Alternatively, virus expression might differ in different ganglia; for example, in ganglia latently infected with HSV, the number of neurons containing HSV DNA is far greater than those revealing latency-associated transcripts (15). Finally, in the subjects whose ganglia contained VZV ORF 63 protein, virus reactivation might have occurred, although this seems unlikely since there was no clinical evidence of zosteriform rash before death; no history of prolonged neuralgic pain, which may precede rash (preherpetic neuralgia) (16); no history of dermatomal distribution pain without rash (zoster sine herpete) (17); and no history of postherpetic neuralgia suggesting a low-grade ganglionitis (18). Furthermore, histologic examination of ganglia revealed no neuronophagia, inclusion bodies, or an inflammatory response.

The VZV ORF 63 protein is an immediate-early *(IE)* gene-encoded protein in virus-infected cells in culture (13). Furthermore, VZV ORF 63 protein has been detected in latently infected rat neurons (13), and we have identified the protein exclusively in the cytoplasm of neurons in latently infected human ganglia. Although VZV does not spontaneously reactivate from rat ganglia, analysis of the physical state of VZV nucleic acid in rat ganglia months after experimental infection might provide useful information about human VZV latency. A comparison of the virus burden during latency in ganglionic neurons of humans and rats cannot yet be made, since our ganglia were obtained from elderly humans, whereas Debrus *et al.* (13) studied ganglia from middle-aged rats months after experimental infection.

The HSV homologue of VZV ORF 63 is HSV-1 IE 68 (19). The HSV-1 IE *68* gene is not transcribed in ganglia latently infected with HSV. In fact, during HSV latency in humans, the only region of the viral genome transcribed is a 2.0-kb fragment of DNA within the repeat regions that synthesizes two RNAs, 2.0 and 1.5 kb in size (20). In this regard, HSV and VZV latency are different.

Finally, the translation products of the three VZV RNAs that have been identified during latency in humans (VZV genes 27, *29,* and *62)* have not been studied, since antibodies directed against these ORFs have not yet been produced. Those virus antigens may be present at a level different from that of VZV ORF 63 protein, or possibly not expressed at all. This is, to our knowledge, the first report of the identification of a herpesvirus protein expressed during latency in the human nervous system. Knowledge of other VZV genes expressed at the peptide level during latency should provoke hypotheses regarding the mechanism(s) of latency.

Aknowledgements

We thank Dr. Bagher Forghani for performing the enzyme-immunoassay studies, Dr. Gregory Owens for useful discussions, Marina Hoffman and Mary Devlin for editorial review, and Cathy Allen for preparing the manuscript. These studies were supported in part by Public Health Service Grants AG 06127 and NS 32623 from the National Institutes of Health.

References

[1]. Mahalingam, R., Wellish, M., Lederer, D., Forghani, B., Cohrs, R. & Gilden, D. H. (1993) *J. Virol* 67, 2381-2384.

[2]. Gilden, D. H., Vafai, A., Shtram, Y., Becker, Y., Devlin, M. & Wellish, M. (1983) *Nature (London)* **306,** 478-480.
[3]. Mahalingam, R., Wellish, M., Wolf, W., Dueland, A. N., Cohrs, R., Vafai, A. & Gilden, D. H. (1990) *N Engl* 7 *Med.* **323,** 627-631.
[4]. Meier, J. L., Holman, R. P., Croen, K. D., Smialek, J. E. & Straus, S. E. (1993) *Virology* **193,** 193-200.
[5]. Cohrs, R. J., Schrock, K., Barbour, M., Owens, G., Mahalingam, R., Devlin, M. E., Wellish, M. & Gilden, D. H. (1994)7. *Virol* **68,** 7900-7908.
[6]. Cohrs, R. J., Barbour, M. B., Mahalingam, R., Wellish, M. & Gilden, D. H. (1995) *J. Virol* **69,** 2674-2678.
[7]. Hyman, R. W., Ecker, J. R. & Tenser, R. B. (1983) *Lancet* ii, 814-816.
[8]. Gilden, D. H., Rozenman, Y., Murray, R., Devlin, M. & Vafai, A. (1987) *Ann. Neurol.* **22,** 377-380.
[9]. Merville-Louis, M. P., Sadzot-Delvaux, C, Delree, P., Piette, J., Moonen, G. & Rentier, B. (1989)7. *Virol.* **63,** 3155-3160.
[10]. Sadzot-Delvaux, C, Merville-Louis, M. P., Delree, P., Marc, P., Piette, J., Moonen, G. & Rentier, B. (1990)7. *Neurosci. Res.* **26,** 83-89.
[11]. Croen, K. D., Ostrove, J. M., Dragovic, L.J. & Straus, S. E. **(1988)** ***Proc. Natl. Acad. Sci. USA*** **85, 9773-9777.**
[12]. Clarke, P., Beer, T., Cohrs, R. & Gilden, D. H. (1995) 7. *Virol* **69,** 8151-8154.
[13]. Debrus, S., Sadzot-Delvaux, C, Nikkels, A. R, Piette, J. & Rentier, B. (1995) 7 *Virol.* **69,** 3240-3245.
[14]. Forghani, B. (1986) in *Methods of Enzymatic Analysis,* ed. Berg-meyer, H. U. (Verlag Chemie, Weinheim, Germany), 3rd Ed., Vol. 10, pp. 267-281.
[15]. Ramakrishnan, R., Levine, M. & Fink, D. J. (1994) 7. *Virol* **68,** 7083-7091.
[16]. Gilden, D. H., Dueland, A. N., Cohrs, R., Martin, J. R., Klein-schmidt-Demasters, D. K. & Mahalingam, R. (1991) *Neurology* 41, 1214-1218.
[17]. Gilden, D. H., Wright, R. R., Schneck, S. A., Gwaltney, J. M., Jr., & Mahalingam, R. (1994) *Ann. Neurol.* **35,** 530-533.
[18]. Mahalingam, R., Wellish, M., Brucklier, J. & Gilden, D. H. (1995) 7. *Neurovirol* 1, 130-133.
[19]. McGeoch, D. J., Dolan, A, Donald, S. & Rixon, F. J. (1985) 7 *Mol Biol* **181,** 1-13.
[20]. Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L. & Feldman, L. T. (1987) *Science* **235,** 1056-1059.