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# Altered expression and changing distribution of the nerve growth associated protein GAP-43 during ocular HSV-1 infection in the rabbit

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**This research examines changes that occur in neurons during corneal herpes simplex virus (HSV-1) infection and focuses on the nerve growth associated protein GAP-43. Cornea and trigeminal ganglion (TG) of New Zealand white rabbits were examined after inoculation of the McKrae and 17 Syn$^+$ strains of HSV-1 to the cornea. Rabbit tissues were taken during acute, latent and induced reactivation stages of infection. Systemic immunosuppression (intravenous injections of cyclophosphamide and dexamethasone) was used to induce reactivation. Western blotting, immunoblotting and autoradiography with the same antibody were used respectively to verify antibody specificity, measure changes in GAP-43 concentration and localize GAP-43 to neurons in the TG. During acute infection, corneal GAP-43 increased significantly while no change was seen in the TG. GAP-43 content was elevated in TG and cornea during viral latency (post-inoculation days 84–154) for both HSV-1 strains. When latent virus was reactivated, the corneal concentration of GAP-43 was more than double that of normal rabbits and the concentration of GAP-43 in TG was reduced compared to the non-reactivated, latently-infected TG. In summary, HSV-1 infected TG neurons expressed more GAP-43 than control neurons and immunosuppressive therapy led not only to viral reactivation and increased GAP-43 concentrations in cornea but also to decreased GAP-43 concentrations in TG. These results suggest that factors which maintain HSV-1 latency and induce reactivation could be linked to elements regulating GAP-43 expression.**

**Keywords:** HSV latency and reactivation; dexamethasone; neuronal immunohistochemistry; sensory ganglia; immunosuppression; cornea

## Introduction

Herpes simplex viruses types 1 and 2 (HSV-1, HSV-2) infections are significant medical problems. Infecting eyes, skin, brain and genitals, the virus can cause serious morbidity and mortality. Primary infections are often asymptomatic and usually occur between the ages of 6 months and 5 years. Following an initial infection, the virus becomes latent in the cell bodies of sensory ganglia (primarily the trigeminal ganglion in the case of ocular HSV-1). Over 90% of clinically significant corneal abnormalities result from reactivation of latent virus. Stress, fever, trauma and immunosuppression can trigger HSV-1 reactivation; however, the likelihood of viral reactivation depends more upon the strain of latent virus than the immunologic status of infected individuals (Kaufman and Rayfield, 1988; Kennedy and Steiner, 1994). For viral latency to exist in an animal model, there must be no virus detected in cell-free tissue samples at the time of sacrifice; yet following a period of culture of explanted tissues, infectious virus must be detectable. The molecular events that establish and maintain viral latency are attributed to the infected neuron (Fawl and Roizman, 1994; Stevens, 1994); however, these molecular mechanisms, like those responsible for reactivating latent virus, are not understood.

The nerve growth-associated protein, GAP-43, is perhaps most strongly implicated in axonal outgrowth during development and in regeneration following axonal damage (Skene and Willard,

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1981a,b), but GAP-43 is also implicated in synaptic plasticity (Fishman and Valenzuela, 1991; Hens *et al*, 1993). In fact, GAP-43 expression is so intimately correlated with these processes that it is often considered an indicator of them (Gispen, 1993). GAP-43 is a protein kinase C substrate that was independently discovered by several investigators and given a different name in each instance, B-50, neuromodulin, F1, P57, pp46 and γ5 (for review see, Coggins and Zwiers, 1991). In the brain, GAP-43 expression is highest in early stages of postnatal development (Jacobson *et al*, 1986). At maturity, the protein is most abundant in pyramidal cells of the hippocampus (Neve *et al*, 1988) and the cerebral cortex (Benowitz *et al*, 1989). GAP-43 expression appears to be constitutive in the peripheral nervous system (Tetzlaff *et al*, 1989; Martin and Bazan, 1992; Stewart *et al*, 1992) but it can be regulated as evidenced by the increased expression seen after injury and during axonal regeneration and sprouting (Meiri *et al*, 1988; Skene and Willard, 1981a). GAP-43 can interact with at least two different second messenger systems, those mediated by inositol lipids (Jolles *et al*, 1980) and those mediated by calmodulin (Andreasen *et al*, 1983; Alexander *et al*, 1987). These interactions are regulated by phosphorylation and intracellular calcium concentrations (for review see Coggins and Zwiers, 1991; Gispen, 1993). GAP-43 could also modulate the activity of certain G-proteins, particularly $G_o$ (Strittmatter *et al*, 1991; Coggins *et al*, 1993).

The cornea and TG provide a unique system in which to study GAP-43 *in vivo* because the cornea is considered one of the most densely innervated epithelia (Rózsa and Beuerman, 1982). We have previously demonstrated a correlation between GAP-43 content and HSV infection in the rabbit in the trigeminal ganglion (TG) following corneal inoculation with HSV-1 (Martin *et al*, 1994) and in dorsal root ganglia following footpad inoculation with HSV-2 in the mouse (Henken *et al*, 1995). Here, using the rabbits infected with either McKrae or 17 $Syn^+$ strains of HSV-1, we examine in greater detail how ocular HSV-1 infection affects GAP-43 expression by measuring changes in the concentration of this protein in the neuronal cell bodies of TG and in their terminal processes in the cornea during the three principal stages of HSV-1 infection, the acute stage (primary infection), the latent stage (when non-replicating virus resides in the TG) and the reactivation stage (when virus begins to replicate in latently infected cells). In this study, systemic immunosuppression induced with intravenous injections of cyclophosphamide (Cx) and dexamethasone (Dx) was used to reactivate latent virus (Stroop and Schaefer, 1986; Rivera *et al*, 1988; Beyer *et al*, 1989, 1990; Haruta *et al*, 1989). We did not want to physically interact with the cornea to induce reactivation because direct stimulation of the nerve fibers could induce GAP-43 expression (Dorster *et al*, 1991; Schreyer and Skene, 1991; Gato *et al*, 1994) and complicate interpretation of the data.

Using a well characterized monoclonal antibody (Schreyer and Skene, 1991), Western blots confirmed absence of (1) detectable antibody cross-reactivity with proteins other than GAP-43 and (2) detectable proteolysis of GAP-43 in the samples. The same monoclonal antibody was also used to immunohistochemically localize GAP-43 to the neuronal cell bodies of TG (data not shown) and measure changes in GAP-43 content by radioimmunoassay of tissue extracts. Some of these data have been reported in abstract form (Martin *et al*, 1994).

## Results

*Western blotting*

Western blotting with monoclonal antibody 91E12 was performed to verify that immunoreactivity seen on immunoblots and in tissue sections corresponded to native GAP-43. Figure 1 shows results of Western blots that were processed using extracts from skeletal muscle (negative control), cornea and TG taken from uninfected control rabbits. The GAP-43 immunoreactivity in these 12% polyacrylamide gels migrated with an apparent molecular weight of 50 kDa. There were no other immunoreactive proteins in these extracts. This result was also seen using tissues from HSV-1 infected rabbits. The results from Western blotting were similar to immunoblots (below) in that skeletal muscle displayed no immunoreactivity. Furthermore, both the Western blots (Figure 1) and the immunoblots (Figures 2 and 3 and Table 1) indicated that, in uninfected rabbits, the immunoreactivity of corneal extracts was always greater than the TG extracts.

*Immunoblotting*

The immune reaction between the monoclonal antibody 91E12 and [$^{125}$I]protein A was used to quantitate the relative enrichment of GAP-43 in extracts of cornea and TG. Immunoblotting experiments reported in Figures 2 and 3 determined GAP-43 content in cornea and TG at different stages of HSV-1 infection with two different virus strains, McKrae and 17 $Syn^+$. Several stages of HSV-1 infection were analyzed: The acute stage (5 dpi), the latent stage (84 dpi and 154 dpi) and the reactivated stage (initiated 6 days prior to sacrifice at 84 dpi and 154 dpi). Assays for GAP-43 immunoreactivity used 2 μg of protein from cornea and TG. These assays were linear between 0.5 and 4 μg of protein ($r$=0.97) for cornea and ($r$=0.99) for TG. Skeletal muscle extracts displayed no immunoreactivity, even when assayed at protein concentrations far exceeding those of cornea and TG (5 μg, 10 μg or 200 μg).

Figure 2 shows changes in the concentration of GAP-43 in cornea and TG of rabbits infected with the McKrae strain of HSV-1. In the acute stage of HSV-1 infection, GAP-43 content was 48% higher than normal in cornea, but not significantly different in the TG. During viral latency, GAP-43 content was elevated in both cornea and TG. At 154 dpi, GAP-43 content was up 104% in TG and 36% in cornea compared to sham-infected animals. Similar results were obtained at 84 dpi. When latent virus was reactivated with Cx/Dx at 154 dpi, corneal GAP-43 content was 72% greater than in the un-reactivated (latent) rabbits and 134% higher than uninfected rabbits. The TG from these rabbits still contained more GAP-43 than control animals but the GAP-43 content was significantly lower (49% at 154 dpi) than that of latently infected animals that were not treated with Cx/Dx to reactivate latent virus.

As with McKrae virus, rabbits latent with HSV-1, strain 17 Syn$^+$ also had elevated GAP-43 concentrations (Figure 3). At 88 dpi (latency) GAP-43 content was elevated 88% and 64% higher than normal in cornea and TG respectively. Reactivating latent 17 Syn$^+$ virus by treating with Cx/Dx at 86 dpi resulted in a further increase in corneal GAP-43 to a level 147% higher than normal. Unlike the TG of rabbits with reactivated McKrae virus, the TG of rabbits with reactivated 17 Syn$^+$ did not contain significantly less GAP-43 than their latently infected, untreated (unreactivated) counterparts. However, the mean GAP-43 content in TG of these rabbits was lower than that of latently infected, untreated rabbits.

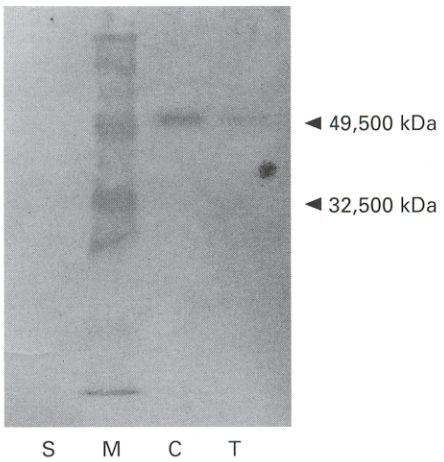


**Figure 1** Western blot analysis of GAP-43 after electrophoresis in 12% sodium dodecylsulfate polyacrylamide gels. The Figure shows an autoradiogram of immunolabeled GAP-43 in register with the nitrocellulose filter from which the autoradiogram was made. The prestained molecular weight standards (M) are visible through the autoradiogram; they were not immunoreactive. The lanes were loaded with 5 $\mu$g of protein from an extract of cornea (C) and 5 $\mu$g of protein from an extract of trigeminal ganglia (T); both the cornea tissue and the TG were taken from the same sham-infected control animal. For a negative control, 10 $\mu$g of skeletal muscle extract was loaded for comparison (S). The tissue extracts and prestained molecular weight standards were electrophoretically transferred to nitrocellulose. The resulting blots were probed with monoclonal antibody 91E12 and [$^{125}$I]protein A. Arrows indicate the molecular weights of the prestained standards.

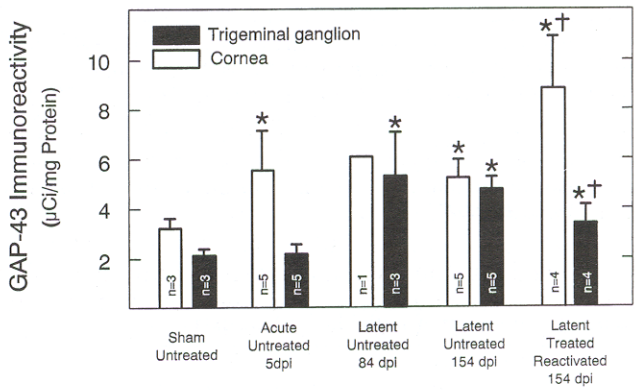


**Figure 2** Quantitative analysis of changing GAP-43 immunoreactivity in cornea and TG of McKrae strain-infected rabbits. Tissue extracts containing 2 $\mu$g of protein were applied to nitrocellulose and probed with monoclonal antibody 91E12 and [$^{125}$I]protein A. Values represent the mean $\pm$ standard deviation obtained from tissue extracts from three or more separate animals unless otherwise indicated. Rabbits that were (1) sham-infected and untreated; (2) acutely-infected, untreated and sacrificed at 5 dpi; (3) latently-infected, untreated and sacrificed at 84 dpi; (4) latently-infected, untreated and sacrificed at 154 dpi; and (5) latently-infected, treated with Cx/Dx to reactivate latent virus and sacrificed at 154 dpi are compared. Differences from sham-infected untreated animals (*) and from 154 dpi, latently-infected untreated rabbits (†) were determined using one-tailed Student's $t$ tests; $P<0.05$ in all cases.

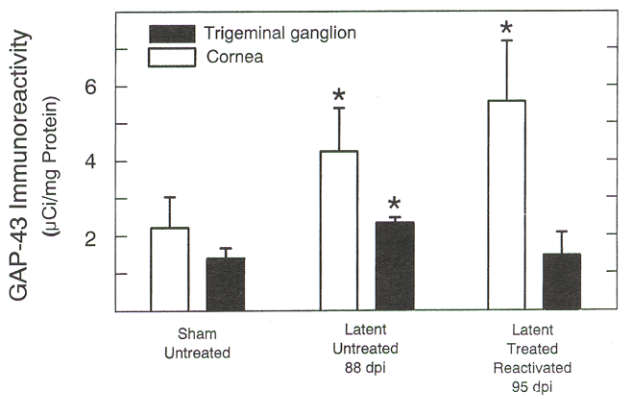


**Figure 3** Quantitative analysis of changing GAP-43 immunoreactivity in cornea and TG of 17 Syn$^+$ strain-infected rabbits. Analyses were conducted as described in Figure 2. Rabbits that were (1) sham-infected and untreated, (2) latently-infected, untreated and sacrificed at 88 dpi, and (3) latently-infected, treated with Cx/Dx to reactivate latent virus and sacrificed at 95 dpi are compared. Values indicate the mean result, $\pm$ standard deviation, that was obtained from three different animals ($n$=3). Differences from sham-infected, untreated animals are indicated (*). These differences were determined using one-tailed Student's $t$ tests; $P<0.05$ in all cases.

Table 1 shows the effect of Cx/Dx treatment in uninfected rabbits. As seen here, when given to sham-infected rabbits, these injections caused no significant change in corneal GAP-43 but the mean GAP-43 content in the TG was reduced. This was evident at both 6 and 19 days after the treatment.

**Table 1** Quantitative analysis of GAP-43 immunoreactivity

| *Condition* | *Tissue* *Cornea* | *Trigeminal Ganglion* |
|---|---|---|
| Control | 2.26±0.82 | 1.43 ±0.28 |
| Cx/Dx+6d | 2.64±0.71 | 0.94[1]±0.28 |
| Cx/Dx+19d | 1.66±0.18 | 0.85[2]±0.24 |

The table compares untreated, sham-infected (control) rabbits with rabbits that were treated with cyclophosphamide (Cx) and dexamethasone (Dx) and sacrificed either 6 days or 19 days later. Values are mean±standard deviation of experiments on three separate rabbits and represent $\mu$Ci[$^{125}$I] protein A/mg protein. Values judged by a one-tailed Student's *t* test as significantly different from the control group are indicated (1, $P$=0.048; 2, $P$=0.027)

## Discussion

Baseline GAP-43 expression by TG neurons is well documented (Erzurumlu *et al*, 1989; Martin and Bazan, 1992) and realizing previous studies indicate HSV-1 latency is correlated with increased GAP-43 in the cornea (Martin *et al*, 1992), we wanted to measure changes in GAP-43 concentration in both the cornea and the TG during the course of HSV-1 infection. Moreover, to demonstrate that our data were not specific for a particular strain of virus, we

**Table 2** Summary of individual rabbit data

| *Rabbit* | *Virus* | *Cx/Dx* | *DPI* | *Days after Cx/Dx* | *Ocular swabs (Virus in tear film)* *Acute* | *Reactivation* | *Slit-lamp evaluations (Epithelial defects)* *Acute* | *Reactivation* | *Classification* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Sham | – | | | N/A | N/A | N/A | N/A | Sham/Untreated |
| 2 | Sham | – | | | N/A | N/A | N/A | N/A | Sham/Untreated |
| 3 | Sham | – | | | N/A | N/A | N/A | N/A | Sham/Untreated |
| 4 | McKrae | – | 5 | | + | N/A | + | N/A | Acute/Untreated |
| 5 | McKrae | – | 5 | | + | N/A | + | N/A | Acute/Untreated |
| 6 | McKrae | – | 5 | | + | N/A | + | N/A | Acute/Untreated |
| 7 | McKrae | – | 5 | | + | N/A | + | N/A | Acute/Untreated |
| 8 | McKrae | – | 5 | | + | N/A | + | N/A | Acute/Untreated |
| 9 | McKrae | + | 84 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 10 | McKrae | – | 84 | | + | – | – | – | Latent/Untreated |
| 11 | McKrae | – | 84 | | + | – | – | – | Latent/Untreated |
| 12 | McKrae | – | 84 | | + | – | – | – | Latent/Untreated |
| 13 | McKrae | + | 154 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 14 | McKrae | + | 154 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 15 | McKrae | + | 154 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 16 | McKrae | + | 154 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 17 | McKrae | + | 154 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 18 | McKrae | – | 154 | | + | – | + | – | Latent/Untreated |
| 19 | McKrae | – | 154 | | + | – | + | – | Latent/Untreated |
| 20 | McKrae | – | 154 | | + | – | + | – | Latent/Untreated |
| 21 | McKrae | – | 154 | | + | – | + | – | Latent/Untreated |
| 22 | Sham | – | | | N/A | N/A | N/A | N/A | Sham/Untreated |
| 23 | Sham | – | | | N/A | N/A | N/A | N/A | Sham/Untreated |
| 24 | Sham | – | | | N/A | N/A | N/A | N/A | Sham/Untreated |
| 25 | 17 Syn$^+$ | – | 88 | | + | – | + | – | Latent/Untreated |
| 26 | 17 Syn$^+$ | – | 88 | | + | – | + | – | Latent/Untreated |
| 27 | 17 Syn$^+$ | – | 88 | | + | – | + | – | Latent/Untreated |
| 28 | Sham | + | | 6 | N/A | N/A | N/A | Normal | Sham/Treated |
| 29 | Sham | + | | 6 | N/A | N/A | N/A | Normal | Sham/Treated |
| 30 | Sham | + | | 6 | N/A | N/A | N/A | Normal | Sham/Treated |
| 31 | 17 Syn$^+$ | + | 95 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 32 | 17 Syn$^+$ | + | 95 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 33 | 17 Syn$^+$ | + | 95 | 5 | + | + | + | + | Latent/Treated/Reactivated |
| 34 | Sham | + | | 19 | N/A | N/A | N/A | Normal | Sham/Treated |
| 35 | Sham | + | | 19 | N/A | N/A | N/A | Normal | Sham/Treated |
| 36 | Sham | + | | 19 | N/A | N/A | N/A | Normal | Sham/Treated |

The table indicates the strain of virus given to each rabbit in the study and whether or not the rabbit was treated with i.v. cyclophosphamide and dexamethasone (Cx/Dx). The number of days which passed after Cx/Dx treatment until sacrifice (Days after Cx/Dx) and between innoculation and sacrifice is indicated (DPI). Evidence of treatment with Cx/Dx, acute infection, latency and reactivation (Classification) is denoted by the presence of virus in the tear film and epithelial defects (+ or –). These determinations were made periodically after inoculation to confirm establishment of latency, each day after inducing reactivation and finally, on the day of sacrifice. Not applicable, N/A

examined rabbits that were infected with either of two different HSV-1 strains, McKrae and 17 Syn$^+$.

To guard against proteolysis of GAP-43 and preserve the extracts, dissected tissues were immediately placed in liquid $N_2$ and stored at $-80°C$. The tissues were ground to powder in a mortar and pestle that was cooled in liquid $N_2$. The powdered frozen tissue was added to a homogenization buffer containing a mixture of metabolic and proteolytic inhibitors. Even so, it was necessary to confirm by Western blotting that immunoreactivity corresponded to native GAP-43. The only immunoreactive protein present in these extracts migrated with an apparent molecular weight of 50 kDa (Figure 1). This was in good agreement with findings of other investigators (Jolles *et al*, 1980) and it demonstrated that there was no detectable proteolysis of GAP-43 or cross-reactivity of the monoclonal antibody with other proteins in the extract.

To compare GAP-43 concentrations in cornea and TG, the tissue extracts were prepared similarly with respect to homogenization, centrifugation and the final ratio of buffer to tissue. Because the cornea has a high collagen content, the low speed corneal pellet was washed with 5 volumes of homogenization buffer to improve separation of the collagenous matrix from membranous material. The analogous pellet from TG was very small in comparison to that of cornea, therefore this wash step was omitted in the extraction of TG.

In all the sham-infected rabbits we examined, the corneal tisuse was more enriched in GAP-43 than the TG (Figures 1, 2 and 3 and Table 1). While this finding could be due to the methods used to extract GAP-43 from these tissues, it is not surprising because GAP-43 is axoplasmically transported to the nerve ending (Goslin and Banker, 1990) and significant GAP-43 immunoreactivity is also present in the densely arborized nerve fibers of rabbit stroma and subepithelial plexus (Martin and Bazan, 1992).

During the acute stage of infection and 5 days after the Cx/Dx treatments, virus was recovered from the tear film of infected rabbits (indicating viral replication in the corneal epithelium), slit-lamp observations indicated typical herpetic epithelial defects (Table 2) and GAP-43 concentrations were elevated in the cornea. During these periods, large portions of the cornea and the nerve endings in it are often damaged (Kaufman and Rayfield, 1988). These corneal nerves can regenerate (Rózsa *et al*, 1983) and both HSV-1 (Rivera *et al*, 1988) and GAP-43 (Tetzlaff *et al*, 1989; Hoffman, 1989) are moved distally by axonal transport. Furthermore, GAP-43 levels increase when neurons are damaged (Dorster *et al*, 1991; Gato *et al*, 1994). Therefore, one could expect to see increased GAP-43 expression during the acute and reactivation stages of HSV-1 infection. Our data confirm this.

Interestingly, during HSV-1 latency (even 154 days after being infected with McKrae virus) the corneas appeared normal by slit-lamp observation (Table 2) but the GAP-43 content of cornea and TG was still elevated compared to sham-infected untreated rabbits (Figures 2 and 3). This elevated GAP-43 content, indicative of neuronal damage, was completely unexpected because during HSV-1 latency, the rabbit corneas appear normal and their response to tactile stimulation is unimpaired (Kaufman and Rayfield, 1988; Beyer *et al*, 1989). Moreover, regeneration of transected rabbit corneal nerves occurs within 90 days (Rózsa *et al*, 1983). The increased GAP-43 seen during latency may be indicative of some sort of long-term damage to the neurons or that some 'fine tuning' of the nerve fibers is still occurring but it may also imply that either HSV-1 infection (latency) is altering GAP-43 expression in the TG or that events related to increasing GAP-43 expression could inhibit replication of the virus and/or potentiate establishment of latency.

Another significant finding of this research was that, during acute HSV-1 infection, GAP-43 was not elevated in the TG and it was elevated in the cornea. Futhermore, when latent McKrae virus was induced to reactivate by treating with Cx/Dx, the GAP-43 content increased in cornea to an extent surpassing that of the untreated, latently-infected rabbits while it decreased in the TG (Figure 2). The unchanged TG GAP-43 content seen during acute infection and the decreased TG GAP-43 content seen after reactivation were unexpected. One explanation for these results could be that when the corneal nerves are altered or insulted, GAP-43 stores shift from proximal locations to the corneal neurite. This issue can be clarified by measuring transcription of GAP-43 mRNA and trafficking of radiolabeled GAP-43 protein under these conditions.

Viral reactivation in the rabbit model can be achieved via a number of different stimuli including localized manipulation of the cornea or systemic injection of classical immunosuppressants (Stroop and Schaefer, 1987; Rivera *et al*, 1988; Beyer *et al*, 1989, 1990; Haruta *et al*, 1989). We chose immunosuppression because we did not want to stimulate changes in GAP-43 content through physical disruption of the corneal nerves. When latently infected rabbits were treated with Cx/Dx, GAP-43 concentrations decreased in the TG (Figures 2 and 3) and latent virus was reactivated. This decrease in TG GAP-43 was unexpected so control experiments were done to evaluate the effects of Cx/Dx in uninfected rabbits. When Cx/Dx was injected in control rabbits, GAP-43 levels decreased in the TG (Table 1). These findings suggest that the decreases in TG GAP-43 content were related to either viral reactivation or to the Cx/Dx treatment itself.

*Summary*
The interactions of HSV-1 with the host cell and mechanisms controlling GAP-43 expression are under intense investigation and there are many findings pertinent to this study. GAP-43 expression is likely regulated by multiple elements that have effect in specific cells at certain times during development (Schryer and Skene, 1991; Reinhard and Skene, 1992). In PC12 cells, GAP-43 transcription is induced by nerve growth factor (NGF) and blocked by Dx (Costello *et al*, 1990; Federoff *et al*, 1988). In agreement with this are our data (Table 1), the results of Chao and McEwen (1994) showing that Dx diminishes GAP-43 in neurons and the fact that systemic anti-NGF injections (which would hypothetically serve to diminish GAP-43 content in the TG) reactivate latent virus in this rabbit model (Hill *et al*, 1992). Taken together, our results and those of others suggest that the program regulating GAP-43 expression in TG neurons is altered when latent HSV-1 is present and that diminishing GAP-43 expression in TG neurons could factor into the reactivation of HSV-1.

## Materials and methods

*Animals and virus*
Adult New Zealand white rabbits were handled and maintained in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research. Viruses were propagated in primary cultures of rabbit kidney cells. African green monkey cells (CV-1) were used to titer the virus by plaque assay. The virus was aliquoted and stored at −70°C. The rabbits were infected bilaterally by applying 25 μl of HSV-1 ($2 \times 10^6$ plaque forming units, McKrae strain or 17 $Syn^+$ strain) to the unscarified corneas (Haruta *et al*, 1989; Bloom *et al*, 1994). Slit-lamp examinations and ocular swabs were done to monitor corneal epithelial defects and virus shedding (Table 2). Rabbits were sacrificed with intravenous injections of pentobarbital and corneas and TG were either immediately frozen in liquid nitrogen for subsequent extraction or prepared for immunohistochemical examination. The rabbits were sacrificed at different days post-infection (dpi) that coincided with different stages of HSV-1 infection: 5 dpi (acute infection studies) and 84, 88, 95 and 154 dpi (latent infection and reactivation studies). Rabbits sacrificed at 5 dpi are referred to as acutely infected because at this time infectious virus was identified in the tear film and the cornea epithelium displayed typical herpetic lesions (Kaufman and Rayfield, 1988). In other experiments, rabbits described as latent were sacrificed at 84 dpi, 88 dpi, 95 dpi and 154 dpi. Ocular swab data (Table 2) indicated that rabbits described as latent had no infectious virus in the affected dermatome (tear film) and the cornea appeared normal as determined by slit lamp observation (Kaufman and Rayfield, 1988; Roizman and Sears, 1987). In studies of viral reactivation intravenous 75 mg/kg injections of Cx followed 24 h later by intravenous 4 mg/kg injections of Dx were given 6 and 5 days respectively prior to sacrifice unless otherwise specified (Tables 1 and 2). This treatment has been shown to reactivate virus in rabbits latently infected with HSV-1 (Haruta *et al*, 1989; Stroop and Schaefer, 1987).

*Preparing extracts*
The tissue samples were transferred from −80°C to liquid nitrogen. While still frozen, the samples were weighed and ground to powder with a mortar and pestle cooled in liquid nitrogen. The freshly powdered tissue was resuspended in homogenization buffer containing 20 mM Tris-HCl, pH 7.5 with 320 mM sucrose, 10 mM ethylene glycol-bis [β-aminoethyl ether] (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 0.05% leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Both corneas from bilaterally infected rabbits were combined to give a single homogenate ($n$=1). One TG from each of these rabbits was examined immunohistochemically and the other was homogenized for immunoblotting ($n$=1). For Western blotting and immunoblotting, corneal tissue was resuspended in 15 volumes of homogenization buffer and TG tissue was resuspended in 20 volumes of homogenization buffer. The homogenates were centrifuged for 10 min at $2000 \times g$ in an Eppendorf microcentrifuge at 4°C to pellet the collagenous stromal matrix and any cellular debris from the TG. The collagenous pellets from corneal homogenates were washed by resuspending them with 5 volumes of buffer and centrifuging as above. This supernatant containing residual GAP-43 from the collagenous pellet was added to the first corneal supernatant to give the final corneal extract. The pellet obtained from the low speed centrifugation of TG homogenate was discarded. The resulting supernatants from cornea and TG were aliquoted and stored at −20°C until further analysis. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard and the Bio-Rad (Richmond, CA) protein assay reagent.

*Electrophoresis/Western blotting*
Proteins (5 μg protein/lane) from cornea and TG were separated in 12% sodium doceclysulfate polyacrylamide (SDS-PAGE) gels following the method of Lamelli (1970). Skeletal muscle, used as a negative control, was loaded at 10 μg protein/lane. The proteins were transferred at 4°C to Hybond ECL nitrocellulose filters (Amersham; Arlington Heights, IL) in buffer containing 25 mM Tris HCl, 192 mM glycine and 20% methanol. Transfer of GAP-43 from the gel to nitrocellulose filters was

done at 4°C for 30–50 min at 50 V constant power using the plate electrode system of Bio-Rad. Following transfer, the nitrocellulose filters were either stained with colloidal gold stain (Bio-Rad) or processed as described below for immunoblotting (Burnette, 1981).

*Immunoblotting GAP-43*

Using the Bio-Rad (Richmond, CA) dot-blot device, 2 μg of protein from the corneal extracts were adsorbed to Hybond-ECL nitrocellulose membranes (Amersham Corp.) presoaked in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% methanol. The GAP-43 content of cornea and TG was measured indirectly by quantitating the immune reaction between GAP-43, a monoclonal antibody to GAP-43 and [$^{125}$I]protein A. The monoclonal antibody 91E12 (Boehringer Mannheim; Indianapolis, IN) is specific for GAP-43 and reacts with both the phosphorylated and dephosphorylated forms of GAP-43 (Jacobsen *et al*, 1986). To block nonspecific binding of monoclonal antibody 91E12, the nitrocellulose filters were incubated for 40–60 min in PBS containing 0.1% polyoxethylenesorbitan monolaurate (Tween 20) and 2% nonfat dry milk (blocking solution). The filters were then incubated in blocking solution containing mouse anti-GAP-43, diluted 1 : 1000, for 1 h. The unbound antibody was removed with three 7 min washes in PBS containing 0.1% Tween 20. The filters were then incubated in blocking solution containing 0.3 mCi/ml [$^{125}$I]protein A (Dupont NEN; Boston, MA). The nonspecifically bound protein A was removed with one rapid wash in blocking solution and three 7 min washes in PBS. The filters were dried and exposed to Hyperfilm β-Max (Amersham Corp.). To further quantify the presence of the GAP-43 immune complex, the corneal blots were cut out of the filter and *bound* [$^{125}$I] was quantitated by scintillation counting. Negative controls for these experiments used skeletal muscle tissue and no immunoreactivity was detected in these samples. Statistical comparisons were based upon blots of many samples (extracts from individual rabbits) that were incubated with a single solution of anti-GAP-43 and [$^{125}$I]protein A. Because extracts prepared from 17 Syn$^+$ and McKrae virus-infected were not assayed with the same solutions of anti GAP-43 and [$^{125}$I]protein A, we did not statistically compare the effects that the two viruses had on GAP-43 levels. We simply compared the effects that progression of the viral infection (with a single strain of virus) had on GAP-43 concentration.

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