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Expression of Multiple Proteins within Single Primary Cortical Neurons Using a Replication Deficient HSV Vector

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ABSTRACT

The study of protein-protein interactions in the nervous system has become dependent on the ability to express foreign proteins (or to overexpress endogenous proteins) within neurons. Often, multiple genes need to be overexpressed in the same cell. To investigate the simultaneous co-expression of more than one virally introduced gene in primary cortical neurons, we infected cultures with two different herpes simplex virus (HSV) vectors and analyzed the proportion of singly and doubly infected cells. The vast majority of neurons expressed both gene products, with a smaller number expressing one or the other protein alone. Increasing the quantity of virus caused an increase in the proportion of doubly labeled cells at the expense of singly labeled cells, which is consistent with a model in which infection with one viral vector is independent of infection with the other. We conclude that co-infection with HSV vectors is an efficient way to obtain expression of multiple gene products within individual primary culture neurons.

INTRODUCTION

Increasingly, infection with recombinant viral vectors is being used for the delivery of genes into primary neuronal cells (1,3,5-7). Often, particularly in the study of in situ protein-protein interactions, an experimental protocol depends on the co-expression of two genes in the same cell. While vectors have been described that express multiple gene products either using separate expression cassettes (4) or as multiple genes driven by the same promoter using an internal ribosome entry site (8), these methods do not allow the combinatorial flexibility afforded by multiple infection with separate vectors. An implicit assumption in co-infection experiments is that the expression of each virally introduced gene is independent of the other; the proportion of singly and doubly infected cells would then be expected to fit a binomial distribution. Alternatively, successful infection of a cell with one viral vector might diminish or even preclude infection with the second vector.

To distinguish between these possibilities, we infected primary neuronal cultures with replication-defective herpes simplex virus (HSV) vectors expressing either a fusion protein of the neuronal growth-associated protein GAP-43 with sGFP (HSVGFPGAP) and/or *Escherichia coli* β -galactosidase (β -gal) (HSVlac). Counts of singly and doubly infected cells suggest that successful infection by each of the two vectors is an independent event, yielding a high proportion of double-infected neurons.

MATERIALS AND METHODS

Generation of Recombinant HSV Vectors

Replication-defective HSV vectors expressing sGFPGAP cDNA or β -gal in the plasmid pHSVPrpUC were prepared as previously described (2,5). The titer of the helper virus component of each stock was $1-1.2 \times 10^6$ plaque-forming units (pfu)/mL on 2-2 cells. The titer of the recombinant virus component of each stock, as assayed by expression of the exogenous gene in PC12 cells, was consistently 3×10^7 infectious units (iu)/mL.

Primary Neuronal Cultures

Primary cortical cultures dissociated from E18 rat embryos were plated at a density of $2-5 \times 10^5$ viable cells per 35 mm dish containing 4 poly-D-lysine coated ACLAR coverslips (Ted Pella, Redding, CA, USA) in neurobasal medium supplemented with B-27 (Life Technologies, Gaithersburg, MD, USA), 1% fetal bovine serum (FBS) and 1% horse serum. After 6-8 days in culture, neurons were infected with HSV vectors diluted to achieve the appropriate MOI and fixed for immunofluorescence after 9-12 h post-infection.

Immunofluorescence and Confocal Microscopy

All steps proceeded at room temperature. Cells were fixed for 45 min in 4% paraformaldehyde, rinsed in PBS, blocked for 30 min in PBS with 10% normal goat serum (NGS) and 0.1% Triton[®] X-100, followed by 2 h of incubation in primary antibody to β -gal (5'-3') at 1:1000 in PBS with 10% NGS and 0.1% Triton X-100. After rinsing in PBS, cells were incubated for 90 min with Cy5[™] goat-anti rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After rinsing in PBS, cells on ACLAR slips were affixed to glass microscope slides, mounted in GelMount (Biomed, Foster City, CA, USA) and covered with glass coverslips. An additional series of infected cultures was triple-labeled with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin to act as a counterstain to visualized uninfected cells. A third series of infected cultures was stained with bis-benzamide to visualize apoptotic nuclei.

Laser scanning confocal microscopy was performed using a TCS-NT system (Leica, Deerfield, IL, USA). Each scanned field was reconstructed from eight optical sections through the Z-plane for each of the two fluorochromes, at a resolution of 1024×1024 pixels, using a $40\times$ 1.25 numerical aperture (NA) Plan Apo oil immersion objective. The use of the fluorochromes sGFP and Cy5, which have completely nonoverlapping emission spectra, and the use of sequential rather than simultaneous scanning, virtually eliminated

the possibility of bleedthrough or cross-excitation of the labels. Additionally, the detection of sGFP_{GAP} by direct fluorescence and the detection of β -gal by immunofluorescence eliminated the possibility of cross-reactivity of two secondary antibodies.

Pseudocolored digital images were then exported to the MCID imaging program for analysis. Thresholding routines were used to define cell boundaries for relative fluorescence intensity measurements in each of the two color channels. For each labeled cell, the ratio $GAP/(GAP + LAC)$ was calculated (where $GAP = sGFP_{GAP-43}$ fluorescence intensity, and $LAC = \beta$ -gal fluorescence intensity), and frequency histograms of this normalized index were constructed across 6–12 40 $\times$ fields for each infection condition. This index was chosen to preserve symmetry in the histograms with a value of approximately 0.5; if $GAP = LAC$, the index has a value of 0.5, if $GAP = 0$, the index has a value of 0, if $LAC = 0$, the index has a value of 1.0. To measure the proportion of infected cells in each field, uninfected cells were identified based on phalloidin-stained actin in their processes and cell bodies.

Bis-benzamide-labeled apoptotic nuclei were quantified by photographing 12 40 $\times$ fields for each infection condition on black-and-white negative film using a DMRB fluorescence mi-

croscope (Leica) with appropriate filters. The negatives were then digitized, and apoptotic nuclei and normal cells were counted using MCID software.

RESULTS AND DISCUSSION

The objective of these experiments was to determine the degree of co-expression of two exogenous genes within individual cells; the protein products of these genes might or might not exhibit subcellular co-localization. Indeed, in many doubly infected cells, sGFP_{GAP} and β -gal showed different subcellular distributions, although both were easily localized to a given cell.

Figure 1 shows that infection rates were determined for singly and doubly infected cultures at an MOI of 0.1, 1.0 and 3.0. The three points representing the double infection conditions (total MOIs of 0.2, 2.0 and 6.0) lie on, or slightly above, the curve representing the single infection conditions. Thus, the probability of a cell being successfully infected (with at least one vector) was dependent on the total MOI, regardless of whether the culture was infected with a single viral vector or both vectors.

We next addressed the question of whether individual cells are singly or doubly infected and the relative expression ratios of the two proteins in single cells. Figure 2 shows representative

Figure 1. The proportion of cells in each field expressing either one or both exogenous proteins as a function of virus concentration. Closed diamonds represent cells infected with HSVGFP_{GAP}, closed squares represent HSV_{lac}, and open circles represent both HSVGFP_{GAP} and HSV_{lac}. Axis label MOI is the total MOI for that condition (i.e., corresponding to infection with one or both vectors).

Short Technical Reports

fields of cells from cultures infected at three different MOIs. Figure 3 shows the corresponding histograms of the relative intensity of the two labels. At the lowest MOI (0.1 for each vector), a few labeled cells are visible in each field. Usually, they are singly labeled, resulting in the broad U-shaped histogram in Figure 3A. At the higher MOIs (1.0, 3.0 for each vector) the ma-

jority of cells expressed both proteins, as seen in Figure 2, leading to a progressive sharpening of the frequency distributions (Figure 3, B and C).

The inverted-U shape of the frequency histograms of doubly and singly labeled cells at higher MOIs, and the finding that a higher MOI shifted the distribution away from single-labeled cells and towards more double-

labeled cells, further support the hypothesis that expression of each virally introduced gene is essentially independent. Furthermore, if expression of both genes is independent, the expected frequency of double-labeled cells would be the simple product of the two individual frequencies, each of which, in turn, would increase as a function of MOI. One can then calculate that as

Figure 2. Confocal dual-fluorescence images of representative fields from cultures co-infected at three different MOIs. MOIs are for each individual vector. GFP (GFP) fluorescence is labeled green; β -gal (lac) immunofluorescence is labeled red. Scale bar = 50 μ m.

Figure 3. Frequency distributions of the ratios of fluorescence intensity in individual cells from cultures infected at different MOIs. (See Materials and Methods for explanation of ratio index). Within each distribution, rightward indicates greater GFP expression and leftward represents greater lac expression.

Short Technical Reports

Figure 4. The proportion of cells in each representative field with bis-benzamide-labeled apoptotic nuclei as a function of virus concentration.

MOI increases, the frequency of double-labeled cells would increase, and the frequency of single-labeled cells would decrease. Indeed, this is the pattern that we observed.

We also measured toxicity as a function of single or double infection using bis-benzamide to label apoptotic nuclei. As previously described (1) we found a low, but dose-related, level of toxicity in singly infected cultures, approximately 5% at an MOI of 1.0 (Figure 4). At each MOI, a comparison of single and double infected cultures revealed a less than additive effect on cell death in the double infected cultures.

The present results demonstrate that double infection of primary cortical cultures with high efficiency HSV vectors yields a large proportion of cells expressing both virally introduced genes. Moreover, by manipulating the levels of the vectors, one can control the ratio of singly and doubly infected cells to achieve a desired range of expression ratios in individual neurons.

Therefore, we believe that this is a reliable method for obtaining high expression of multiple gene products within a defined population of cells, specifically suited for in situ studies of protein-protein interactions.

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