The $\gamma_134.5$ gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells

(virulence/gene expression/apoptosis/human cells/tissue-specific genes)

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ABSTRACT The $\gamma_134.5$ gene of herpes simplex virus 1 was previously shown to play a role in viral virulence since deletion of the gene reduced by a factor of $\approx 100,000$ the capacity of the virus to replicate in the central nervous system and cause mortality in the mouse. Here we show that in the human neuroblastoma cell line SK-N-SH of neuronal origin $\gamma_134.5$ null mutants expressed early proteins, viral DNA, and mRNA of late genes. However, the onset of viral DNA synthesis triggered complete cessation of incorporation of radioactive precursors into proteins. The mutant and wild-type viruses replicated and could not be differentiated in cell lines or cell strains of nonneuronal origin. The results indicate that in the absence of the $\gamma_134.5$ gene the SK-N-SH neuroblastoma cells triggered a response similar to the programmed cell death of neuronal cells induced by metabolic stress. The $\gamma_134.5$ protein precludes this cell response possibly in order to enable the protein synthesis necessary for viral replication.

The most severe and devastating manifestation of herpes simplex virus 1 (HSV-1) infection is encephalitis, which afflicts ≈ 1 in 250,000 adults (1). Of considerable interest in connection with this disease are the genes that confer upon the virus the capacity to replicate in the central nervous system (CNS) tissues. Chou *et al.* (2) reported that a viral gene designated as $\gamma_134.5$ enables the virus to multiply and spread in the CNS of mice even though it is not essential for viral replication in cell culture and in at least some murine tissues tested to date (R. J. Whitley, E. R. Kern, S. Chatterjee, J.C., and B.R., unpublished data). The $\gamma_134.5$ gene is located in the inverted repeats flanking the long component of viral DNA and therefore the gene is present in two copies per genome (4–7) (see Fig. 1). Its location is consistent with previous reports showing that a locus that affected the capacity of the virus to multiply in the CNS tissues mapped at the right end of the long component of HSV DNA in the prototype arrangement (2, 8–11).

In the course of studies designed to determine the function of the $\gamma_134.5$ gene product, we discovered that infection of cells of neuronal origin with mutants incapable of expressing the $\gamma_134.5$ gene resulted in shutoff of cellular protein synthesis, whereas infection of cells of nonneuronal origin with wild-type or mutant viruses resulted in sustained protein synthesis and production of infectious progeny. (Some of the results reported in this article were presented at the 15th International Herpesvirus Workshop, July 7–12, 1991, Asilomar, CA.)

MATERIALS AND METHODS

Cells. Vero cells originally obtained from the American Type Culture Collection (ATCC) were propagated in Dulbecco's modified Eagle's medium (DME medium) containing

FIG. 1. Schematic representation of the genome structure and sequence arrangements of the HSV-1(F) and related mutants. Line 1: The two covalently linked components of HSV-1 DNA, L and S, each consist of unique sequences flanked by inverted repeats (7, 13). The reiterated sequences flanking the L component designated as ab and b'a' are each 9 kilobases (kb) in size, whereas the repeats flanking the S component are 6.3 kb in size (13). Line 2: Expansion of portions of the inverted repeat sequences ab and b'a' containing the $\gamma_134.5$ and α_0 genes. Line 3: Sequence arrangement and restriction endonuclease sites in the expanded portions shown on line 2. The open box represents the 20-base-pair (bp) direct repeat sequence (DR1), flanking the "a" sequence (14, 15). Restriction site designations are N, *Nco* I; Be, *Bst*EII; S, *Sac* I; St, *Stu* I. Line 4: The thin line and filled rectangle represent the transcribed and coding domains of the $\gamma_134.5$ gene (4–6). The vertical line indicates the location of the transcription initiation sites of $\gamma_134.5$ and α_0 genes. In the R3616 viral recombinant, 1 kb was deleted between *Bst*EII at the 28th amino acid of $\gamma_134.5$ to *Stu* I at the 3' terminus of the genes as shown. In HSV-1(F)R DNA, the sequences deleted from the $\gamma_134.5$ gene in R3616 were restored, and therefore the virus could be expected to exhibit a wild-type phenotype. The R4009 recombinant virus DNA contains an in frame translation termination codon at the *Bst*EII site. The vertical arrow on top points to the site of the stop codon insertion. The constructions and biologic properties of the wild-type and recombinant viruses were reported elsewhere (2).

5% calf serum. The human SK-N-SH neuroblastoma (NB) cell line was obtained from the ATCC (HTB11) and propagated in DMEM containing 10% fetal bovine serum.

Viruses. The isolation of HSV-1 strain F [HSV-1(F)] (12) has been described elsewhere. The construction of recombinant viruses R3616, R4009, and HSV-1(F) was reported elsewhere (2). As illustrated in Fig. 1, R3616 contains a 1-kilobase-pair (kbp) deletion in both copies of the $\gamma_134.5$ gene. In the recombinant R4009 a stop codon was inserted in both copies of the $\gamma_134.5$ gene. The $\gamma_134.5$ genes in the recombinant R3616 were restored to yield the recombinant HSV-1(F)R.

Virus Infection. Cells were generally exposed to the viruses for 2 hr at 37°C at a multiplicity of infection of 5 and then removed and replaced with the 199v medium containing 1% calf serum. The infection continued at 37°C for the length of time indicated for each experiment. Cells were then either labeled for *de novo* protein synthesis or analysis of viral DNA and RNA.

[³⁵S]Methionine Labeling. At the indicated time after infection, 50 μ Ci of [³⁵S]methionine (specific activity, >1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) was added to 1 ml of 199v medium lacking methionine to cells in a six-well dish. Labeling was continued for 1.5 hr, at which time cells were harvested. Preparation of cell extracts, separation of proteins by electrophoresis in denaturing polyacrylamide gels crosslinked with *N,N'*-diallyltartardiamide (Bio-Rad), transfer of polypeptides to nitrocellulose sheets, autoradiography, and immunoblot with antibodies have been described (16).

RESULTS

HSV-1 Recombinant Viruses Lacking the γ 34.5 Gene Induce the Shutoff of Protein Synthesis in NB Cells. In the course of screening human cell lines derived from CNS tissues we noted that the SK-N-SH NB cell lines produced 100-fold less mutant viruses than the fully permissive Vero cells. We also noted, as shown in Fig. 2, that the SK-N-SH NB cells infected with R3616 or with R4009 exhibited reduced protein synthesis in cells harvested at 7 hr (Fig. 2 *Left*) and ceased to incorporate [³⁵S]methionine by 13 hr (Fig. 2 *Right*) after

infection. The phenomenon was observed in SK-N-SH NB cells only and could be attributed specifically to the mutations in the γ 34.5 gene inasmuch as restoration of the deleted sequences yielded a virus [HSV-1(F)R] that expressed viral proteins at 13 hr after infection (Fig. 2 *Right*) and exhibited the parental, wild-type phenotype (2).

The Shutoff of Protein Synthesis Occurred After the Expression of α Genes. Viral genes form three major groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (18). The α genes do not require *de novo* protein synthesis for their expression, the β genes, which are required for the synthesis of viral DNA, require prior synthesis of functional α proteins for their expression, and the γ genes require for full expression α and β proteins and the onset of viral DNA synthesis. To determine the point at which expression of viral gene functions was terminated in SK-N-SH NB cells infected with mutant viruses, infected cell lysates electrophoretically separated in denaturing polyacrylamide gels were transferred to a nitrocellulose sheet and probed with antibody to an α (α 27) (16, 18), a β (viral thymidine kinase), and two abundant γ proteins. The latter were the products of U_L26.5 (19, 20) and of U_S11 genes (21) whose expression at optimal levels requires viral DNA synthesis. As shown in Fig. 3, the SK-N-SH NB cells infected with the mutant viruses made normal amounts of α 27 protein (Fig. 3 *Left*), reduced amounts of the thymidine kinase (β) protein (Fig. 3 *Middle*), but no detectable γ proteins (Fig. 3 *Left* and *Right*). In contrast, the wild-type and mutant viruses could not be differentiated with respect to their capacity to

FIG. 2. Autoradiographic images of electrophoretically separated lysates of infected cells labeled for 90 min with [³⁵S]methionine at stated time points. The SK-N-SH NB and Vero cell lines were mock infected (M) or exposed at 37°C to 5 plaque-forming units (pfu) of wild-type or mutant viruses in six-well (Costar) dishes. At 2 hr after exposure the cells were overlaid with mixture 199 supplemented with 1% calf serum. At 5.5 and 11.5 hr after exposure of cells to viruses, replicate infected six-well cultures were overlaid with 1 ml of the 199v medium lacking unlabeled methionine but supplemented with 50 μ Ci of [³⁵S]methionine (specific activity, >1000 Ci/mmol; Amersham). After 90 min in labeling medium, the cells were harvested, solubilized in a buffer containing sodium dodecyl sulfate, subjected to electrophoresis on a denaturing 12% polyacrylamide gel crosslinked with *N,N'*-diallyltartardiamide, electrically transferred to a nitrocellulose sheet, and subjected to autoradiography as described (16). Infected cell polypeptides (ICP) were designated according to Honess and Roizman (17) and Morse *et al.* (22).

Fig. 3. Autoradiographic images of labeled polypeptides electrophoretically separated in denaturing gels and photographs of protein bands made apparent by their reactivity with antibodies. The SK-N-SH NB and Vero cells were either mock infected (M) or infected with 5 pfu of either R3616 or the parent HSV-1(F) per cell as described in the legend to Fig. 2. The cultures were labeled for 1.5 hr before harvesting at 13 hr after exposure of cells to virus. Preparation of cell extracts, electrophoresis of the polypeptides, electric transfer of the separated polypeptides to a nitrocellulose sheet, and autoradiography were carried out as described (16). The nitrocellulose sheets reacted with the respective antibodies with the aid of kits from Promega according to manufacturer's instruction. Monoclonal antibodies H1142 against $\alpha 27$ (22) and H725 against the product of the $U_L 26.5$ gene (19) were the generous gift of Lenore Pereira (University of California, San Francisco). The M28 monoclonal antibody reacts with the $U_S 11$ protein (21) and the rabbit polyclonal antibody R161 against viral thymidine kinase (βtk) were made to a specific peptide (M. Sarmiento and B.R., unpublished studies) in this laboratory. ICP designations, see legend to Fig. 2.

replicate or to direct the synthesis of their proteins in Vero cells (Fig. 3).

The Signal for Shutoff of Protein Synthesis Is Linked to Viral DNA Synthesis. These experiments were designed to determine whether the shutoff of protein synthesis was linked to a gene whose expression was dependent on viral DNA synthesis. The results of a key experiment are shown in Fig. 4. Replicate SK-N-SH and Vero cell cultures were infected with HSV-1(F) and recombinant viruses and maintained in the presence or absence of inhibitory concentrations of PAA, a drug that blocks viral DNA synthesis. The cells were pulse labeled with [35 S]methionine at 13 hr after infection. The salient feature of the results is that protein synthesis in SK-N-SH cells infected with either R3616 or R4009 was sustained for at least 13 hr in the presence of PAA but not in its absence. These results indicate that the signal for cessation of protein synthesis in SK-N-SH NB cells infected with mutant viruses was associated with viral DNA synthesis or with a γ gene dependent on viral DNA synthesis for its expression.

Human NB Cells Infected with the $\gamma_{134.5}$ Mutants Synthesized Viral DNA and Accumulated Late mRNA Even Though the Shutoff of Protein Synthesis Precluded Accumulation of Late Proteins. The evidence presented above indicated that in SK-N-SH NB cells an event associated with viral DNA synthesis triggered the shutoff of protein synthesis and that the late (γ) viral proteins did not accumulate. We expected, therefore, little or no accumulation of viral DNA and, in the absence of viral DNA synthesis, little or no accumulation of late (γ) viral transcripts whose synthesis is dependent on viral DNA synthesis. To our surprise, the amounts of viral DNA recovered from SK-N-SH NB cells 17 hr after infection with mutant viruses were comparable to those obtained from the wild-type parent or repaired [HSV-1(F)R] viruses (Fig. 5 Left). Furthermore, although the SK-N-SH NB cells did not

synthesize demonstrable amounts of $U_S 11$ protein, the amounts of $U_S 11$ gene transcripts that accumulated in cells infected with mutant and wild-type viruses were of similar magnitude (Fig. 5 Right).

DISCUSSION

The significance of our results stems from three observations. First, in infected cells, protein synthesis reflects a regulatory cascade; α protein synthesis is replaced by β and later by γ protein synthesis. In all cell lines other than the SK-N-SH NB cells infected with the $\gamma_{134.5}$ mutants and tested to date, a block in the synthesis of one group of proteins does not lead to a cessation of total protein synthesis. For example, in cells treated with inhibitors of DNA synthesis such as PAA, a subclass of γ proteins dependent for their synthesis on viral DNA synthesis is not made. However, in these cells, β protein synthesis continues beyond the time of their synthesis in untreated infected cells. The striking observations made in the studies on SK-N-SH cells infected with the $\gamma_{134.5}$ null mutants are that (i) all protein synthesis ceased completely, (ii) viral DNA was made, and (iii) late, γ mRNA exemplified by $U_S 11$ mRNA was made even though protein synthesis ceased. These manifestations of viral replication have not been observed previously and are not characteristic of cells infected with wild-type virus or any mutant virus infection of cells (e.g., Vero, HEp-2, baby hamster kidney, 143tk⁻, and rabbit skin cell lines and human embryonic lung cells) other than those described in this report.

Second, the function of the $\gamma_{134.5}$ gene is to overcome this block in protein synthesis in SK-N-SH cells since repair of the mutation restores the wild-type phenotype.

Last, although the association of cessation of protein synthesis with the onset of viral DNA replication does not exclude the possibility that a product made after infection is

FIG. 4. Autoradiographic image of viral proteins expressed during infection on SK-N-SH NB cell lines in the presence or absence of phosphonoacetate (PAA). Duplicate SK-N-SH NB cell cultures were either treated with PAA (300 μ g/ml; Sigma) starting at 1.5 hr prior to infection continuously until the termination of the infection or left untreated. The cultures were either mock infected (M) or exposed to 5 pfu of HSV-1(F), R3616, R4009, or HSV-1(F)R at 5 pfu per cell. At 11.5 hr after exposure to virus, the cells were overlaid with medium containing 50 μ Ci of [35 S]methionine as described in the legend to Fig. 2. Polypeptide extraction, electrophoresis on 12% polyacrylamide gels crosslinked with N,N' -diallyltartardiamide, electrical transfer to nitrocellulose sheets, and autoradiography were as described in the legend to Fig. 2. ICP designations, see legend to Fig. 2.

responsible of the shutoff, the data do support the hypothesis that the cessation of protein synthesis is specifically caused by a known viral gene product interacting with the protein synthesizing machinery of the cell. For example, it has been well established that the product of the HSV-1 gene designated as *vhs* can shut off cellular protein synthesis after infection (25–27). *vhs* is a structural protein of the virus and is introduced into cells during infection. It destabilizes mRNA early in infection and its effects are not dependent on viral gene products made after infection (27). In experiments reported here, protein synthesis of wild-type and mutant viruses could not be differentiated at 13 hr after infection in cells treated with PAA and hence the phenotype of mutant viruses cannot be attributed to the *vhs* gene product. This conclusion is reinforced by the observation that viral protein synthesis in SK-N-SH cells was not affected by increasing the multiplicity of infection with wild-type virus to values as high as 100 pfu per cell (data not shown). A more likely source for the genetic information for the cessation of protein synthesis is the cell itself.

It has been reported that deprivation of growth factors from cells of neuronal origin results in programmed cell death, which manifests itself initially by the cessation of protein

FIG. 5. Viral DNA and RNA accumulation in infected SK-N-SH NB and Vero cell cultures. (Left) Photograph of ethidium bromide-stained agarose gel containing electrophoretically separated *Bam*HI digests of total DNAs extracted from mock-infected cells or cells infected with HSV-1(F), R3616, R4009, or HSV-1(F)R virus. (Right) Hybridization of electrophoretically separated RNA transferred to a nitrocellulose sheet probed with RNA sequences antisense to α 47, U_{S10} , and U_{S11} open reading frames. SK-N-SH NB and Vero cells were either mock infected or exposed to 5 pfu of HSV-1(F) or R3616 per cell. Total DNAs were extracted from cells at 17 hr after infection by the procedure published by Katz *et al.* (23), digested with *Bam*HI, electrophoresed in a 0.8% agarose gel at 40 V overnight, and stained with ethidium bromide for visualization. For RNA analysis, SK-N-SH NB and Vero cells were either mock infected or infected with R3616 and HSV-1(F) as described above. At 13 hr after exposure of cells to virus the RNA was extracted by the procedure of Peppel and Baglioni (24). The RNAs were then separated by electrophoresis on a 1.2% agarose gel, transferred by gravity to a nitrocellulose sheet, and probed with antisense RNA made from *in vitro* transcription of pRB3910 (21) off T7 promoter using a kit from Promega according to manufacturer's instruction. α 47, U_{S10} , and U_{S11} transcripts overlap in sequence and share the same 3' coterminal sequence (20). The U_{S10} transcript is of low abundance and is not detected in this assay.

synthesis and subsequently by fragmentation of DNA (28–30). Apoptosis in lymphocytes is manifested by degradation of DNA (31). In the case of other herpesviruses, it has been shown that in B lymphocytes infected with the Epstein–Barr virus, the product of the viral LMP-1 gene induces the host gene Bcl-2, which precludes programmed lymphocyte death (32). It is conceivable that the onset of viral DNA synthesis in neuronal cells triggers programmed cell death by cessation of protein synthesis and that HSV-1 γ 134.5 gene precludes this response.

The evolution of a HSV-1 gene that would preclude a response to a neuronal stress is not surprising. Infection of neurons, especially sensory neurons, is an essential feature of viral reproductive life-style that enables the HSV-1 to remain latent and to survive in human populations. If, as we propose, the function of γ 134.5 is to preclude programmed cell death, the target of the gene would be neurons rather than lymphocytes since HSV does normally infect these cells.

The γ 134.5 gene has several unusual features. The gene lacks a conventional TATAA box or response elements frequently associated with TATAA-less transcriptional units. The sequence that enables the expression of the gene is 12 bp long but is repeated as many as 23 times in the wild-type strain used in this laboratory (refs. 14 and 15, unpublished data). Various assays reported elsewhere indicate that the amounts of gene products produced in cells of nonneuronal derivation are smaller than those expressed by most viral genes and that the amounts of the protein made in the absence of viral DNA synthesis were smaller than those made in its presence (2, 5, 6). The gene is predicted to encode a protein of 263 amino

acids (6). It contains the triplet Ala-Thr-Pro repeated 10 times and accumulates in the cytoplasm (5). A recent note (31) indicates that 63 amino acid residues near the carboxyl terminus of the $\gamma_134.5$ protein share 83% identity with a mouse protein MyD116 found in a myeloid leukemic cell line induced to differentiate by interleukin 6 (3, 33). The function of MyD116 is unknown. In the studies presented here we showed that the product of the $\gamma_134.5$ gene quite clearly enables sustained protein synthesis in SK-N-SH NB cells. Although the mechanism by which it acts is not known, we suspect that it is designed to preclude the cessation of protein synthesis rather than to contribute a factor necessary for the protein synthesizing machinery.

Further experiments should resolve many questions regarding the function of the $\gamma_134.5$ gene. The one answer that is clear is the reason why the mutants were avirulent even when injected directly into mouse CNS at very high concentrations: in the absence of protein synthesis there could be no replication or spread of viral progeny.

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