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Genetics

Characterization of a herpes simplex virus type 1 mutant resistant to benzhydrazone, a selective inhibitor of herpesvirus glycosylation

(syncytium-inducing mutant/complementation/intertypic recombinants)

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ABSTRACT **Benzhydrazone [BH; 1*H*-benz[*f*]indene-1,3(2*H*)-dionebis(amidinohydrazone)] significantly inhibits glycosylation of proteins, but only in cells infected with herpes simplex virus. We report on a herpes simplex virus type 1 (HSV-1) mutant resistant to BH. A syncytium-inducing mutant designated HSV-1(13)S11 was found to be biochemically resistant to BH in that [^{14}C]glucosamine incorporation was not inhibited in infected HEp-2 cells exposed to the drug. Intertypic recombinants were obtained which showed that BH resistance is encoded in the DNA of the mutant virus and may be transferred into the genome of BH-sensitive HSV. In the recombinants the biochemical resistance marker segregated from the syncytial marker, suggesting that the two markers probably map in different loci. The BH-resistant mutant did not complement wild-type BH-sensitive HSV-1 and -2. Furthermore, resistance was apparent in HEp-2 but not in Vero cells. The paper discusses the hypothesis that inhibition of glycosylation of HSV proteins is the consequence of modification or selective transport of BH involving a HSV gene product.**

An earlier report showed that a bis(amidinohydrazone) derivative [1*H*-benz[*f*]indene-1,3(2*H*)-dionebis(amidinohydrazone)], designated benzhydrazone (BH), inhibits glycosylation of the major herpes simplex virus type 1 (HSV-1) glycoproteins (1, 2). BH appears to be a selective inhibitor of herpesvirus glycosylation in that it does not affect HSV-1 protein synthesis or the glycosylation of uninfected cells, Sindbis virus, or paramyxovirus proteins (2). Studies on glycopeptide size-distribution and endo-β-*N*-acetylglucosaminidase H sensitivity of HSV glycoproteins formed in the presence of BH indicated that the compound prevents high-mannose oligosaccharide addition to proteins (3). BH causes, therefore, an early block in the asparagine-linked N-glycosylation process similar to that induced by tunicamycin. Along with glycosylation, BH inhibits herpesvirus-induced cell fusion and reduces the yield of infectious virus (2).

The observation that glycosylation of all HSV glycoproteins may be selectively inhibited by BH suggested that HSV DNA encodes a function that activates BH, or permits its penetration into the infected cells, or participates in glycosylation of viral proteins. If this were the case, it would be expected that mutants resistant to BH might occur and should be selectable. The objective of these studies was to test this hypothesis. Among several mutants tested, the syncytium-inducing (Syn$^-$) mutant HSV-1(13)S11 appeared to be BH-resistant (BH^R) in that the compound did not inhibit incorporation of [^{14}C]glucosamine into infected cells. By constructing intertypic recombinants, we observed that BH resistance is encoded in HSV-1(13)S11 DNA and may be transferred from the genome of the resistant mutant into the genome of HSV-2(G).

MATERIALS AND METHODS

Materials. BH was synthesized as previously described (1).

Viruses and Cells. The BH-resistant (BH^R) mutant HSV-1(13)S11 is a Syn$^-$ mutant selected from HEp-2 cell cultures infected with stocks obtained after 5-bromodeoxyuridine mutagenesis of the non-syncytium-inducing (Syn$^+$) HSV-1(13) (4); isolation and selection were as described for HSV-1(13)B4 (5). HSV-1(HFEM)H6 was described (6). HSV-1(Meinz) is a Syn$^-$ human isolate received from M. H. Wolf (Institute of Medical Microbiology and Immunology, University of Bonn). Growth and infection of cells and exposure to BH were as described (2, 3). Briefly, the inhibitor was added to cell culture medium from the end of virus adsorption until harvesting, in a final concentration of 30 μM. Multiplicity of infection was 10–20 plaque-forming units per cell. In mixed infection experiments, cells were infected simultaneously with 10 plaque-forming units of each virus per cell.

Glycoprotein and Protein Synthesis. Labeling media for glycoproteins and proteins consisted, respectively, of minimal essential medium containing ½ the usual concentration of glucose and [^{14}C]glucosamine (2–3 μCi/ml, 50 mCi/mmol, the Radiochemical Center, Amersham; 1 Ci = 37 GBq) or minimal essential medium containing $\frac{1}{10}$ the usual concentration of methionine and [^{35}S]methionine (25 μCi/ml, >400 Ci/mmol, New England Nuclear). Glycoproteins were labeled between 14 and 20 hr after infection. α polypeptides were detected by exposing cells to cycloheximide (5 μg/ml) from 1 hr before virus adsorption to 5 hr after infection and by labeling for 20 min after cycloheximide removal. β and γ polypeptides were labeled from 5 to 7 and from 15 to 18 hr after infection, respectively. Protein nomenclature was according to Morse *et al.* (7). Details for harvesting cells and measuring trichloroacetic acid-precipitable radioactive material were described (2). NaDodSO$_4$ electrophoresis of glycoproteins and proteins was on 8.5% polyacrylamide gels crosslinked with *N*,*N*′-diallyl tartardiamide.

Intertypic Marker Transfer. Intertypic marker transfer was done essentially as described (8, 9). Approximately 1 μg of HSV-1(13)S11 DNA digested with *Hin*dIII was mixed with 0.5 μg of intact HSV-2(G) DNA and the mixture was used to transfect rabbit skin cells. The cultures were then incubated for about 5 days. Titers of progeny virus were determined on HEp-2 cells. Recombinants that exhibited Syn$^-$

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Abbreviations: HSV-1 and -2, herpes simplex virus type 1 and type 2; Syn$^-$, syncytium-inducing; Syn$^+$, non-syncytium-inducing; BH, benzhydrazone [1*H*-benz[*f*]indene-1,3(2*H*)-dionebis(amidinohydrazone)]; BH^R and BH^S, benzhydrazone-resistant and -sensitive.

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plaques were selected from the viral progeny of transfected cells and were plaque purified three more times before preparing the virus stocks. Purification and digestion of DNAs cut with restriction endonucleases were as described (9).

RESULTS

Selection of a BH^R Mutant: HSV-1(13)S11. We analyzed several Syn$^-$ HSV-1 mutants to identify a BH^R mutant. We took advantage of the observation that BH, as an inhibitor of HSV glycosylation, prevents cell fusion induced by Syn$^-$ mutants of HSV. Therefore, as a criterion in search for BH^R mutants, we looked for Syn$^-$ mutants retaining the Syn$^-$ phenotype when infected cells were exposed to the compound. Among the Syn$^-$ mutants tested, one designated HSV-1(13)S11, isolated from mutagenized stock of HSV-1(13) wild-type (Syn$^+$) virus (4, 5), caused cell fusion in the presence of the drug. Appearance of polykaryocytes was only retarded by BH by 1–2 hr. Subsequent studies showed that the drug did not significantly inhibit [^{14}C]glucosamine incorporation in HSV-1(13)S11-infected HEp-2 cells labeled from 12–14 to 20 hr after infection—i.e., after onset of fusion in both untreated and BH-treated cultures (Table 1). The parental Syn$^+$ HSV-1(13) was sensitive to inhibition by BH, though to a lower extent than HSV-1(MP) (2), which was included as a BH-sensitive (BH^S) control and for comparison (Table 1). It is of interest, however, that HSV-1(13)S11 expressed the resistant phenotype in HEp-2 cells and not in Vero cells (data not shown).

Analyses of the Glycoproteins and of the Polypeptides Specified by HSV-1(13)S11 and Its Parent HSV-1(13). Fig. 1 shows that the electrophoretic pattern of HSV-1(13)S11 glycoproteins synthesized in the absence of BH did not differ from that of glycoproteins synthesized in the presence of the drug. Unlike the parental HSV-1(13), glycoprotein C does not accumulate in HSV-1(13)S11-infected cells, as is frequently the case in Syn$^-$ mutants, which fuse HEp-2 cells (10).

Fig. 2 shows the electrophoretic pattern of polypeptides synthesized in HEp-2 cells infected with HSV-1(13)S11 and with parental HSV-1(13) and labeled between 5 and 7 or 15 and 18 hr after infection. The significant conclusion is that the BH does not alter the pattern of protein synthesis either in the HSV-1(13) parent strain or in the HSV-1(13)S11 mutant, in accordance with previous data (2). However, significant differences were observed between the polypeptides synthesized by the parental and the mutant strains. The main ones concerned infected cell polypeptides 15, 23, 32, and 35, which were present in detectable amounts only in HSV-1(13)-infected cells. These proteins belong to the subset of γ proteins made late in infection and dependent on viral DNA synthesis for their expression (11). The electrophoretic profiles of α polypeptides specified by the parental and the mutant strain did not differ (data not shown). No differences were observed in the patterns of β proteins. The subset of early γ proteins (e.g., ICP 5) dependent on DNA synthesis for normal yields but not for the initiation of their synthesis was only slightly reduced. Whether absence of some polypeptides in HSV-1(13)S11-infected cells is relevant to the expression of the BH^R phenotype remains to be determined.

Table 1. Effect of BH on [^{14}C]glucosamine incorporation and on morphological phenotype in HEp-2 cells infected with various HSV-1 strains

Virus	[^{14}C]Glucosamine incorporation: cpm/μg of protein, No BH	[^{14}C]Glucosamine incorporation: cpm/μg of protein, With BH	[^{14}C]Glucosamine incorporation: % inhibition	Morphological phenotype, No BH	Morphological phenotype, With BH
HSV-1(MP)	970	243	75	Syn$^-$	Syn$^+$
HSV-1(13)	610	280	54	Syn$^+$	Syn$^+$
HSV-1(13)S11	573	527	8	Syn$^-$	Syn$^-$
HSV-1(H6)	844	287	66	Syn$^-$	Syn$^+$
HSV-1(Meinz)	705	399	43	Syn$^-$	Syn$^+$

HEp-2 cells were infected with 10 plaque-forming units per cell of each strain and labeled with [^{14}C]glucosamine from 14 to 20 hr after infection.

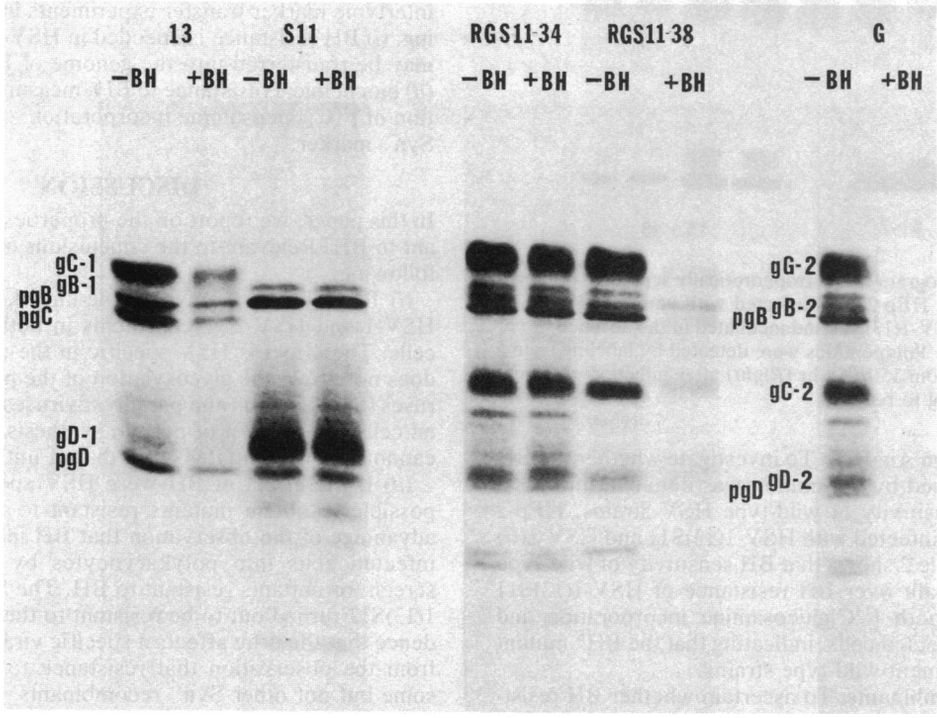

FIG. 1. Autoradiogram of electrophoretically separated glycoproteins synthesized in HEp-2 cells infected with parental HSV-1(13), BH^R mutant HSV-1(13)S11, recombinants RGS11(34) and RGS11(38), and HSV-2(G) and incubated in the absence (−) or presence (+) of BH. Glycoprotein nomenclature is according to the rules agreed upon at the Herpesvirus Workshop, Oxford, July 1983.

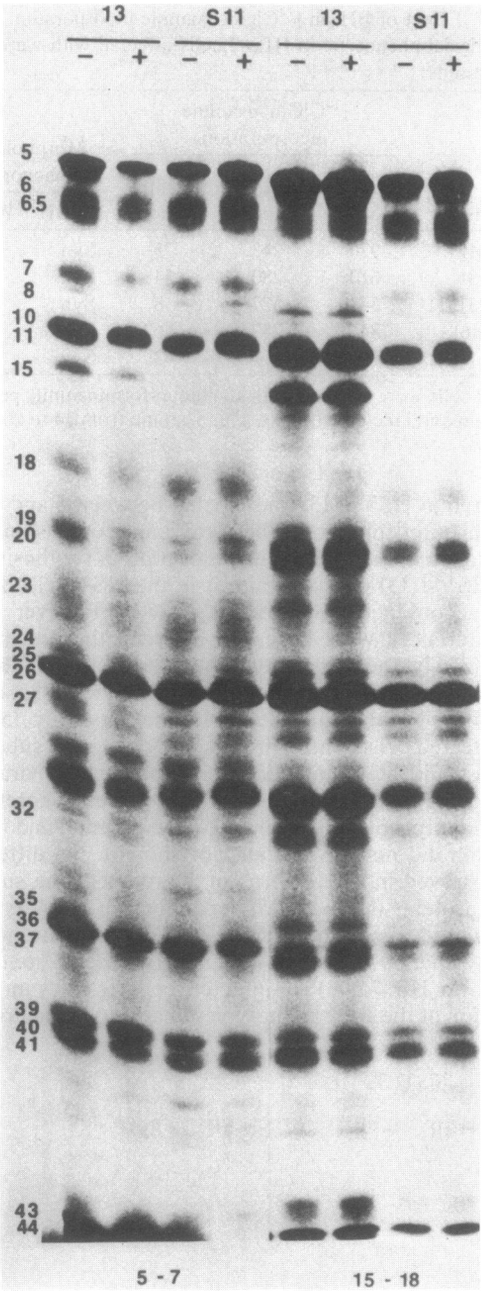

FIG. 2. Autoradiogram of electrophoretically separated polypeptides synthesized in HEp-2 cells infected with parental HSV-1(13) and BH^R mutant HSV-1(13)S11 and incubated in the absence (−) or presence (+) of BH. Polypeptides were detected by labeling from 5 to 7 hr (*Left*) and from 15 to 18 hr (*Right*) after infection and were designated according to ref. 7.

Complementation Analysis. To investigate whether the BH resistance expressed by the mutant was dominant or recessive over BH sensitivity of wild-type HSV strains, HEp-2 cells were doubly infected with HSV-1(13)S11 and HSV-1(F) or HSV-2(G). Table 2 shows that BH sensitivity of wild-type HSVs was dominant over BH resistance of HSV-1(13)S11 with respect to both [^{14}C]glucosamine incorporation and morphology of infected cells, indicating that the BH^R mutant could not complement wild-type strains.

Intertypic Recombinants. To ascertain whether BH resistance was a viral function encoded in HSV-1(13)S11 DNA that might be transferred into the genome of recipient BH^S HSV strains, we performed intertypic marker transfer experiments by transfecting rabbit skin cells with mixtures of

Table 2. Effect of BH on [^{14}C]glucosamine incorporation and morphological phenotype in HEp-2 cells doubly infected with BH^R HSV-1(13)S11 and BH^S HSV-1(F) or HSV-2(G)

HSV	[^{14}C]Glucosamine incorporation cpm/μg of protein: No BH	With BH	% inhibition	Morphological phenotype with or without BH*
(13)S11	229	202	12	Syn^-
F	274	130	53	Syn^+
(13)S11 + F	248	100	60	Syn^+
G	175	49	72	Syn^+
(13)S11 + G	218	55	75	Syn^+

*Morphological phenotype did not differ in the absence or presence of BH.

*Hin*dIII-digested HSV-1(13)S11 DNA and intact HSV-2(G) DNA. The progeny of individual Syn^- plaques were harvested, purified, and analyzed with restriction endonucleases. The electrophoretic pattern of recombinants DNA could not be differentiated from that of HSV-2(G) when cut with *Kpn* I (Fig. 3) or *Eco*RI, *Hin*dIII, *Xba* I, or *Hpa* I (data not shown). Twelve intertypic recombinants were then assayed for expression of biochemical resistance to BH. Whereas all recombinants expressed the Syn^- phenotype in HEp-2 cells in both the absence and the presence of BH, some recombinants were found to be BH^R in that [^{14}C]glucosamine incorporation was not inhibited by BH and some recombinants were found to be BH^S in that [^{14}C]glucosamine incorporation was inhibited by BH. Fig. 1 illustrates the effect of BH on the electrophoretic pattern of glycoproteins synthesized by a BH^R recombinant [RGS11(34)] and by a BH^S recombinant [RGS11(38)]. The electrophoretic patterns of the DNAs of only two recombinants are presented because all other recombinants yielded similar results. The effect of BH on [^{14}C]glucosamine incorporation of HSV-2(G)-infected cells is also shown in Fig. 1. It should be noted parenthetically that HSV-2(G) was reproducibly more sensitive to inhibition by BH than were HSV-1 isolates. The results of intertypic marker transfer experiments indicated the following: (*i*) BH resistance is encoded in HSV-1(13)S11 DNA and may be transferred into the genome of BH^S HSV-2(G) and (*ii*) biochemical resistance to BH, measured as lack of inhibition of [^{14}C]glucosamine incorporation, segregated from the Syn^- marker.

DISCUSSION

In this paper, we report on the properties of a mutant resistant to BH. Relevant to the conclusions of this paper are the following:

(*i*) BH reduces the asparagine-linked N-glycosylation of HSV-1 and HSV-2 glycoproteins in both HEp-2 and Vero cells. The effect is HSV specific in the sense that the drug does not affect the glycosylation of the proteins of other viruses (Sindbis virus and paramyxoviruses). In HSV-1-infected cells, the pattern of protein synthesis in treated cells (2) cannot be differentiated from that of untreated cells.

(*ii*) If the effect of BH were HSV specific, it should be possible to obtain mutants resistant to the drug. We took advantage of the observation that BH inhibits the fusion of infected cells into polykaryocytes by Syn^- mutants to screen for mutants resistant to BH. The Syn^- mutant HSV-1(13)S11 turned out to be resistant to the drug. Further evidence that the drug affects a specific viral function emerged from the observation that resistance to BH segregated in some but not other Syn^- recombinants. The association of BH resistance with the Syn^- marker is not clear. Although some HSV-1 × HSV-2 Syn^- recombinants were sensitive to BH, and hence it could be concluded that Syn^- and BH^R markers segregate, it should be stressed that BH^R mutants

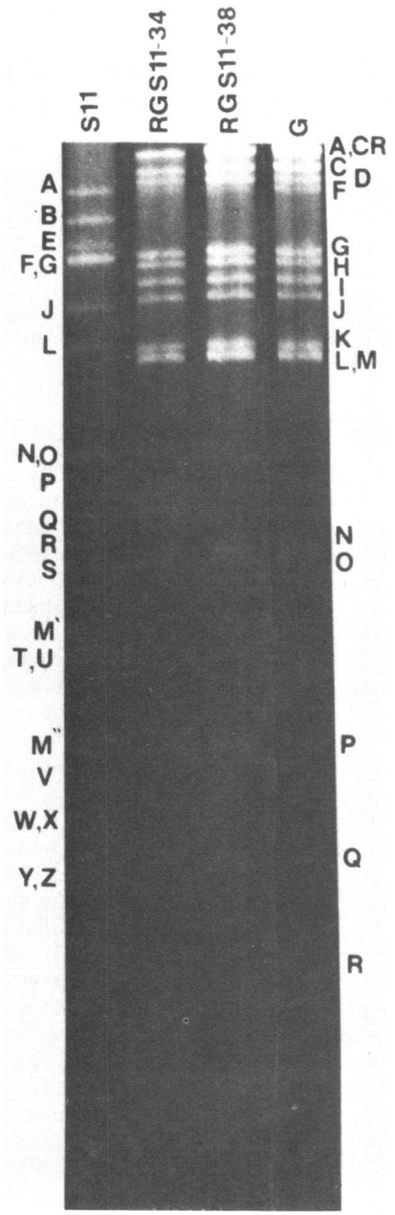

FIG. 3. Photographs of electrophoretically separated *Kpn* I digests of HSV-2(G), HSV-1(13)S11, and two of their recombinants produced by marker transfer. Fragments were designated according to ref. 12.

were selected on a Syn^- background, that Syn^+ BH^R mutants were not sought, and that HSV-1 Syn^+ × HSV-2 Syn^+ crosses occasionally yield Syn^- recombinants.

(*iii*) In principle, drug-resistant mutants fall into two categories. The first, exemplified by HSV thymidine kinase-negative mutants, consists of mutations that result in a loss of function. A characteristic of such mutations is that, in cells doubly infected with wild-type and mutant viruses, the wild type is dominant (13). The second category, exemplified by HSV phosphonoacetate-resistant mutants, involves no loss of function but rather a modification of the gene product such that it no longer interacts with the drug. In cells doubly infected with wild-type and phosphonoacetate-resistant mutants, the mutant is dominant (14). The resistance to BH expressed by the HSV-1(13)S11 mutant is recessive in cells doubly infected with mutant and wild type. The data suggest, therefore, that the mutation in HSV-1(13)S11 resulted in a loss of a function. Consistent with this conclusion is the observation that the synthesis of several late γ proteins (γ_2 proteins), which are stringently dependent on the onset of replication of viral DNA for their synthesis, is reduced or delayed.

(*iv*) A consideration of the nature of the viral functions affected by BH must take in consideration both the phenotype of the resistant mutant HSV-1(13)S11 and the observation that resistance is expressed in HEp-2 cells but not in Vero cells. Two hypotheses could explain our observations. The first hypothesis envisions that BH must be activated by a viral protein for its inhibitory effect and that the mutation alters the affinity of the protein for the drug in HEp-2 cells but not in Vero cells. There are other examples of chemicals (e.g., nucleoside analogues) that must be altered (e.g., phosphorylated) by a viral protein for their effect. Cell dependence for the expression of mutant phenotypes is well known. For example, Syn^- mutants may differ considerably among themselves with respect to the range of cells they fuse (8, 15). The second hypothesis is that uninfected cells are not readily permeable to BH but infected cells become permeable as a consequence of modifications of cellular membranes induced by late viral gene products. This hypothesis envisions that the mutation in HSV-1(13)S11 delays the modifications of cellular membranes beyond the time of maturation of viral glycoproteins. The hypothesis predicts that the membrane modifications occur earlier or at lower dosage of viral gene products in Vero cells than in HEp-2 cells. Studies reported elsewhere that inhibition of [^{14}C]glucosamine incorporation is expressed late in the reproductive cycle (2) do not differentiate between the two hypotheses. Neither hypothesis bears on the question whether HSV specifies enzymes involved in glycosylation of its proteins, inasmuch as the activated BH could act exclusively on cellular enzymes. Differentiation between the two hypotheses rests on studies measuring the entry and modification of BH in uninfected and infected cells.

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