Neutralization of Herpes Simplex Virus Ribonucleotide Reductase Activity by an Oligopeptide-Induced Antiserum Directed against Subunit H2

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Herpes simplex virus type 1 ribonucleotide reductase is associated with two polypeptides of apparent molecular weights 136,000 and 38,000. The two polypeptides form a tight complex and, therefore, are often coprecipitated by monoclonal antibodies. We report here that immunoglobulins G purified from polyclonal rabbit antisera (P9) raised against a nonapeptide corresponding to the carboxy terminus of the 38,000-dalton polypeptide specifically neutralize the herpes simplex virus ribonucleotide reductase activity. We suggest that the P9 immunoglobulin G neutralizes the reductase activity by impairing the association of the two subunits (H1 and H2) of the enzyme.

Ribonucleotide reductase, an essential enzyme for the synthesis of deoxyribonucleotides, is formed by the association of two nonidentical subunits in almost all procaryotic and eucaryotic cells. Suggestive evidence recently obtained by different approaches indicates that the same model probably holds for the herpes simplex virus (HSV)-encoded ribonucleotide reductase. First, it has been shown that chemicals which specifically inactivate each one of the two subunits of the mammalian ribonucleotide reductase also inactivate the HSV reductase (6). Second, polyclonal antiserum raised against a partially purified preparation of viral reductase or a series of monoclonal antibodies which immunoprecipitate or neutralize the enzymatic activity immunoprecipitate two viral proteins of molecular weights 144,000 and 38,000 (4, 13). These polypeptides have also been designated Vmw 136' and Vmw 38' for HSV type 1 (HSV-1) and Vmw 138' and Vmw 36' for HSV type 2 (HSV-2) (10, 19). Their association in the immunoprecipitate is thought to be due to a tight binding essential for an active enzymatic complex (3, 4, 10). However, as some monoclonal antibodies reacted with both proteins by immunoprecipitation and by immunoblotting assays (12), the existence of a related epitope(s) could not be ruled out. Third, genetic studies on a thermosensitive mutant which, at the permissive temperature, induces a reductase activity that was thermosensitive both *in vivo* and *in vitro* have shown that the 136,000-dalton (136K) protein is an essential component of the viral enzyme (8, 19). Fourth, comparisons of the DNA-coding sequences of the 136K and 38K proteins with the sequences of, respectively, the subunit 1 and 2 genes from other species (*Escherichia coli*, Epstein-Barr virus, mouse, and clam) have revealed significant stretches of homology at the amino acid sequence level (20, 23).

However, as attempts to separately purify the two polypeptides were unsuccessful in numerous laboratories (2, 3, 6), final direct proof of the involvement of these peptides in reductase activity is still to be obtained. With a view to obtain better proof of the involvement of the 38K polypeptide in the HSV ribonucleotide reductase activity, we raised sera directed against a synthetic peptide corresponding to

the carboxy-terminal nine amino acids of this polypeptide (11) and analyzed their reactions with polypeptides of HSV-infected cells and their effects on cellular and viral reductase activities.

Propagation of BHK 21/C13 and 96-V-2(600) cells (16) and production of HSV-1 (strain F) and HSV-2 (strain HG52) on BHK 21/C13 cells have been previously described (6, 15). Cellular and viral ribonucleotide reductase extracts were partially purified from, respectively, exponentially growing 96-V-2(600) cells and serum-starved BHK-21/C13 cells infected with HSV-1 or HSV-2 (6, 18). Rabbits were immunized, as described by Benoit et al. (5), with a conjugate of bovine serum albumin and the nonapeptide NH₂-Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu-COOH synthesized by the solid-phase technique of Merrifield (17). Antipeptide antibodies were detected in antisera (designated P9) by radioimmunoassay after 8 weeks (data not shown). The ability of these antibodies to recognize HSV-1 38K and HSV-2 36K polypeptides was determined by immunoprecipitation experiments. Monolayers of BHK-21/C13 cells mock infected or infected with HSV-1 or HSV-2 at 20 PFU/cell were labeled between 0 and 7 h after infection with 100 μ Ci of [³⁵S]methionine. Cell extracts were prepared as described by Suh et al. (22), and the immunoprecipitation was carried out according to the procedure of Kessler (14) except that the immunoglobulin G (IgG) was adsorbed with a 20% suspension of IgG Sorb (The Enzyme Center, Boston, Mass.).

As expected, material immunoprecipitated was primarily polypeptides of 38K from HSV-1-infected cells and 36K from HSV-2-infected cells (Fig. 1A, lanes 3 and 4). Additional species of molecular weights 136,000 and 138,000, for HSV-1- and HSV-2-infected cells, respectively, were specifically immunoprecipitated but much less efficiently. Preimmune rabbit serum did not react with any of these polypeptides (Fig. 1A, lane 1). The immunoprecipitation assay was also done with the monoclonal antibody Bg7 which is known to interact with HSV-2 36K polypeptide and to coprecipitate the 138K polypeptide (3). In addition, this monoclonal neutralizes the HSV-2 ribonucleotide reductase (4). The results showed that antibodies Bg7 and P9 precipitated polypeptides of identical molecular weights (Fig. 1B, lanes 1 and 2 for HSV-2; data not shown for HSV-1).

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FIG. 1. Specificity of P9 antinonapeptide antiserum. (A) HSV-1- or HSV-2-infected and mock-infected BHK-21/C13 cells were labeled with [35 S]methionine from 0 to 7 h postinfection and harvested at 7 h. Cell lysate preparation, immunoprecipitation, and sodium dodecyl sulfate-9% polyacrylamide gel electrophoresis were done as described in the text. Lanes 2, 3, 4, Mock-, HSV-1-, and HSV-2-infected cell lysates, respectively, reacted with P9 immune rabbit serum; lane 1, HSV-1-infected cell lysate reacted with preimmune rabbit serum. (B) Immunoprecipitations were performed with Bg7 monoclonal antibody (lane 1) or P9 antiserum (lane 2).

However, the P9 antiserum precipitated the 138K polypeptide less efficiently than did the Bg7 monoclonal antibody, the 36K being equally immunoprecipitated. Strong evidence that the HSV-2 36K polypeptides reacting with the two antibodies were identical was provided by the finding that the 36K protein immunoprecipitated first by the P9 antiserum could be reimmunoprecipitated by the Bg7 monoclonal antibodies after elution from the appropriate area of a preparative gel (data not shown).

In an attempt to understand if the presence of the 136K polypeptide in the P9 antiserum was the result of a specific immunological reaction or of a coprecipitation, we analyzed the reaction of the P9 antiserum with HSV-1-infected crude cell lysates and with partially purified HSV-1 ribonucleotide reductase preparation by the immunoblotting technique, essentially according to the procedure of Towbin et al. (25). With 100 μ g of HSV-1-infected cell lysate, only the 38K antigen could be detected (Fig. 2A, lane 3). However, a reaction with the 136K polypeptide could be observed when a minimum of 50 μ g of partially purified HSV-1 ribonucleotide reductase was loaded on the gel instead of the crude lysate (Fig. 2A, lanes 4 to 7). At high protein concentration, probable degradation products of the 38K polypeptide were detected (Fig. 2A, lanes 6 and 7). Preimmune serum did not react with either the 38K or the 136K polypeptide (Fig. 2A, lane 1). Moreover, removal of anti-bovine serum albumin antibodies from the P9 antiserum by adsorption with bovine serum albumin did not modify the immunological reaction pattern (data not shown).

To investigate the possibility of a related epitope between HSV-1 38K and 136K polypeptides, we first demonstrated that only the anti-peptide antibodies were responsible for the immunological reaction with these two polypeptides by probing immunoblots of partially purified, HSV-1-infected cell extracts with the P9 antiserum extensively adsorbed with the nonapeptide. This adsorption prevents the detection of both the 136K and the 38K antigens (Fig. 2B, lane 2). These results suggest strongly that the antibodies directed

against the nonapeptide are responsible for both reactions. The second and most convincing approach was to elute the P9 antibodies specifically bound to 38K antigen immobilized to nitrocellulose paper as described by Smith and Fisher (21). These specific antibodies were then used to reprobe a parallel immunoblot of partially purified HSV reductase preparation. The eluted anti-38K antibodies reacted with both the 38K and the 136K antigens (Fig. 2B, lane 3), demonstrating that the antibodies reacting with the 38K polypeptide were able to recognize the 136K polypeptide.

The effects of IgG purified from preimmune or P9 sera on viral and cellular ribonucleotide reductase activities are illustrated in Fig. 3. As little as 2 μ g of P9-purified IgG diminished HSV-1 reductase activity by 50%, and complete inhibition was obtained with 10 μ g of IgG. The neutralizing ability was specific for the anti-peptide antibodies as, first, no inhibition was observed with the preimmune serum (Fig. 3); second, the IgGs eluted from the 38K band of a preparative immunoblot were neutralizing (data not shown); and third, addition of 20 μ M peptide to 10 μ g of IgG prior to the reaction with the protein extract resulted in a reduction of only 10% of the enzyme activity (Fig. 3). Cellular ribonucleotide reductase purified from reductase-overproducing 96-V-2(600) cells or BHK-21/C13 cells was not inhibited by the IgG purified from the P9 antiserum (Fig. 3; data not shown for BHK-21/C13). HSV-2 ribonucleotide reductase activity was neutralized to the same extent by the antinonapeptide antibodies, confirming the cross-reactivity of the P9 antiserum. These results demonstrate clearly that the anti-peptide antibodies neutralize specifically the HSV-ribonucleotide reductase.

Our demonstration that the antinonapeptide antibodies

FIG. 2. Evidence of a related epitope between HSV-1 136K and 38K polypeptides. (A) Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper. The paper was incubated with P9 rabbit antiserum (diluted 1/500) except in lane 1. Lane 2, Partially purified BHK-21/C13 cell extract (100 μ g); lane 3, HSV-1-infected cell lysate (100 μ g); lanes 4 (2 μ g), 5 (25 μ g), 6 (50 μ g), and 7 (100 μ g), given amounts of partially purified HSV-1-infected cell extract. Lane 1, 100 μ g of partially purified HSV-1-infected cell extract was reacted with preimmune rabbit antiserum (diluted 1/500). (B) A 100- μ g portion of partially purified HSV-1-infected cell extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose paper. The paper was cut into strips, and each strip was incubated with: lane 1, P9 immune rabbit serum (diluted 1/500); lane 2, P9 immune rabbit serum extensively adsorbed with the nonapeptide; lane 3, eluted anti-38K antibodies.

which have reacted with the 38K polypeptide are able to recognize in a second-step immunoblot the 136K polypeptide could be explained by the presence of a related epitope on the 136K polypeptide. That these antibodies are considerably less reactive towards the 136K polypeptide can be explained by a partial amino acid sequence homology between the shared epitopes or by a less accessible internal epitope contrasting with a surface-oriented carboxy-terminal sequence. It is generally believed that protein epitopes cover five to seven amino acid residues which often are not consecutive (conformational epitopes). However, there is increasing evidence for the existence of short sequential epitopes, particularly at the protein carboxy terminus (26). Such epitopes could be provided by the linear sequence of only three amino acids of the carboxyl end, as demonstrated by Wehland et al. (27) for the antitubulin monoclonal antibody YL1/2. By computer-assisted analysis, we searched for related epitopes shared by our nonapeptide and the HSV-2 138K polypeptide and found that the sequences Ala-Val-Val and Ala-Gly-Ala were present one and two times, respectively, in the 138K polypeptide. Even if they are not particularly striking, these homologies could be considered as potential related antigenic determinants. The short size of our nonapeptide will facilitate further investigation on this matter.

The existence of related epitopes between the two polypeptides has been previously suggested to explain why two monoclonal antibodies, 6-A6 and 6-H11, reacted in immunoblot assays with the two polypeptides of the HSV-2 reductase (12, 23). As these antibodies predominantly recognized, respectively, the 136K or 38K polypeptide, we can assume that they reacted with two different epitopes. Therefore, the two HSV reductase polypeptides seem to share several related epitopes, the significance of which is currently unknown. However, the recent intriguing observation that several monoclonal antibodies reacting with the B2 subunit polypeptides of *E. coli* ribonucleotide reductase also recognized the B1 subunit polypeptides in immunoblots sheds new light on these related epitopes (1). Hence, as these monoclonal antibodies did not react in solution with the native B1 polypeptide, it was hypothesized that a change(s) in the conformation of the denatured protein bound to the nitrocellulose paper was responsible for this unexpected reactivity. Since all antibodies directed against the HSV reductase coprecipitate its two subunit polypeptides, it is difficult at this time to determine if the HSV reductase polypeptides must be denatured to reveal related epitopes. However, Bacchetti et al. (3; S. Bacchetti, personal communication) have obtained results suggesting that this could be the case for the related epitope recognized by monoclonal antibody 6-H11.

The most important characteristic of our P9 antibodies is their neutralizing properties. From the data presented here we cannot completely rule out that the slight reaction of our P9 antiserum with the 136K polypeptide could be responsible for this property. However, as this reaction on immunoblot was at least 20-fold lower than with the 38K polypeptide, it seems unlikely that it could significantly contribute to the neutralization of enzymatic activity. In addition, we and others recently found that the nonapeptide itself is a specific inhibitor of HSV ribonucleotide reductase activity and suggested that the carboxy terminus of the 38K polypeptide is involved in the binding to the 136K polypeptide (7, 9). This hypothesis was recently substantiated by our finding that the presence of the nonapeptide during immunoprecipitation done with Bg7 antibodies substantially re-

FIG. 3. Neutralization of HSV-1 ribonucleotide reductase activity by P9 antiserum. Enzyme preparations partially purified from HSV-1-infected BHK-21C/13 (40 μ g with a specific activity of 27 U/mg) (●) or 96-V-2(600) (40 μ g with a specific activity of 34 U/mg) (○) cells were incubated for 30 min at room temperature with increasing concentrations of IgG purified as previously described (13) from rabbit antipeptide or preimmune serum (Δ). Ribonucleotide reductase activity was then assayed and expressed as the percentage of activity measured in the reaction without IgG. (■) represents the reductase activity obtained when 20 μ M nonapeptide was added to 10 μ g of IgG prior to the assay. Neutralization of HSV-2 ribonucleotide reductase activity by P9 antiserum gave comparable results.

duced the coprecipitation of the 138K polypeptide (unpublished data). Therefore, it is tempting to speculate that the binding of P9 antibodies to the 38K carboxy terminus prevents the correct association of the two subunits and by doing so is directly responsible for neutralization of the enzymatic activity. The smaller amount of 138K polypeptide in the immunoprecipitates with P9 antibodies as compared with those with Bg7 antibodies favors this interpretation. The residual detection of the 138K polypeptide in the precipitate obtained with P9 antibodies could be due to the previously discussed related epitope or to the presence of residual complexed polypeptides.

Experiments are in progress to obtain more direct proof that our antinonapeptide antibodies could inhibit the enzyme activity by impairment of the binding of the two subunits of HSV reductase. Strikingly, four anti-B1 and three anti-B2 monoclonal antibodies, which neutralize *E. coli* ribonucleotide reductase, all interfered with the interaction between the two enzyme subunits (1). Moreover, antitubulin monoclonal antibody YL1/2 which reacts with the carboxy terminus of the M2 subunit and neutralizes cellular reductase activity (24), might also act by interference with the correct association of the two enzyme subunits. Therefore, it is tempting to speculate that the interaction between the two HSV

ribonucleotide reductase subunits (H1 and H2) is a very attractive target for the production of an antiviral agent.

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LITERATURE CITED

- Anderson, A., T. Barlow, E. Pontis, and P. Reichard. 1986. Production and characterization of monoclonal antibodies against the two subunits proteins B1 and B2 of *Escherichia coli* ribonucleotide reductase. *Biochemistry* 25:860-867.
- Averett, D. R., C. Lubbers, G. B. Elion, and T. Spector. 1983. Ribonucleotide reductase induced by herpes simplex type 1 virus. Characterization of a distinct enzyme. *J. Biol. Chem.* 258:9831-9838.
- Bacchetti, S., M. J. Eveleigh, and B. Muirhead. 1986. Identification and separation of the two subunits of the herpes simplex virus ribonucleotide reductase. *J. Virol.* 57:1177-1181. (Erratum, *J. Virol.* 58:713, 1986.)
- Bacchetti, S., M. J. Eveleigh, B. Muirhead, C. S. Sartori, and D. Huszar. 1984. Immunological characterization of herpes simplex virus type 1 and 2 polypeptide(s) involved in viral ribonucleotide reductase activity. *J. Virol.* 49:591-593.
- Benoit, R., P. Böhlen, N. Ling, A. Briskin, F. Esch, P. Brazeau, and R. Guillemin. 1982. Presence of somatostatin-28-(1-12) in hypothalamus and pancreas. *Proc. Natl. Acad. Sci. USA* 79:917-921.
- Cohen, E. A., J. Charron, J. Perret, and Y. Langelier. 1985. Herpes simplex virus ribonucleotide reductase induced in infected BHK-21/C13 cells: Biochemical evidence for the existence of two non-identical subunits, H1 and H2. *J. Gen. Virol.* 66:733-745.
- Cohen, E. A., P. Gaudreau, P. Brazeau, and Y. Langelier. 1986. Specific inhibition of herpesvirus ribonucleotide reductase by a nonapeptide derived from the carboxy terminus of subunit 2. *Nature (London)* 321:441-443.
- Dutia, B. M. 1983. Ribonucleotide reductase induced by herpes simplex virus has a virus-specified constituent. *J. Gen. Virol.* 64:513-521.
- Dutia, B. M., M. C. Frame, J. H. Subak-Sharpe, W. C. Clark, and H. S. Marsden. 1986. Specific inhibition of herpesvirus ribonucleotide reductase by synthetic peptides. *Nature (London)* 321:439-441.
- Frame, M. C., H. S. Marsden, and B. M. Dutia. 1985. The ribonucleotide reductase induced by herpes simplex virus type 1 involves minimally a complex of two polypeptides (136K and 38K). *J. Gen. Virol.* 66:1581-1587.
- Galloway, D. A., and M. A. Swain. 1984. Organization of the left-hand end of the herpes simplex virus type 2 *Bgl*IIIN fragment. *J. Virol.* 49:724-730.
- Goldstein, L. C., L. Corey, J. K. McDougall, E. Tolentino, and R. C. Nowinski. 1983. Monoclonal antibodies to Herpes simplex viruses: use in antigenic typing and rapid diagnosis. *J. Infect. Dis.* 147:829-837.
- Huszar, D., S. Beharry, and S. Bacchetti. 1983. Herpes simplex virus-induced ribonucleotide reductase: development of antibodies specific for the enzyme. *J. Gen. Virol.* 64:1327-1335.
- Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen. *J. Immunol.* 115:1617-1624.
- Langelier, Y., and G. Buttin. 1981. Characterization of ribonucleotide reductase induction in BHK-21/C13 Syrian hamster cell line upon infection by herpes simplex virus (HSV). *J. Gen. Virol.* 57:21-31.
- Lewis, W. H., and P. R. Srinivasan. 1983. Chromosome-mediated gene transfer of hydroxyurea resistance and amplification of ribonucleotide reductase activity. *Mol. Cell. Biol.* 3:1053-1061.
- Merrifield, B. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149-2154.
- Ponce de Leon, M., R. J. Eisenberg, and G. H. Cohen. 1977. Ribonucleotide reductase from herpes simplex virus (type 1 and 2) infected and uninfected KB cells: properties of the partially purified enzymes. *J. Gen. Virol.* 36:163-173.
- Preston, V. G., J. W. Palfreyman, and B. M. Dutia. 1984. Identification of a herpes simplex virus type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase. *J. Gen. Virol.* 65:1457-1466.
- Sjöberg, B.-M., H. Eklund, J. A. Fuchs, J. Carlson, N. M. Standart, J. V. Ruderman, S. J. Bray, and T. Hunt. 1985. Identification of the stable free radical tyrosine residue in ribonucleotide reductase. A sequence comparison. *FEBS Lett.* 183:99-102.
- Smith, D. E., and P. A. Fisher. 1984. Identification, developmental regulation and response to heat shock of antigenically related forms of a major nuclear envelope protein in drosophila embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell. Biol.* 99:20-28.
- Suh, M., A. Kessous, N. Poirier, and R. Simard. 1982. Immunoprecipitation of polypeptides from hamster embryo cells transformed by herpes simplex virus type 2. *Virology* 104:303-311.
- Swain, M. A., and D. A. Galloway. 1986. Herpes simplex virus specifies two subunits of ribonucleotide reductase encoded by 3'-coterminal transcripts. *J. Virol.* 57:802-808.
- Thelander, M., A. Gräslund, and L. Thelander. 1985. Subunit M2 of mammalian ribonucleotide reductase. Characterization of a homogeneous protein isolated from M2-overproducing mouse cells. *J. Biol. Chem.* 260:2737-2741.
- Towbin, H., H. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
- Van Regenmortel, M. H. V. 1984. Molecular dissection of antigen by monoclonal antibodies, p. 43-82. In N. J. Stern, and H. R. Gamble (ed.), *Hybridoma technology in agricultural and veterinary research*. Rowman and Allanheld, Totowa, N.J.
- Wehland, J., H. C. Schröder, and K. Weber. 1984. Amino acid sequence requirement in the epitope recognized by the α -tubulin-specific rat monoclonal antibody YL1/2. *EMBO J.* 3:1295-1300.