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Identification and Separation of the Two Subunits of the Herpes Simplex Virus Ribonucleotide Reductase

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The herpes simplex virus ribonucleotide reductase is associated with two viral proteins which are both immunoprecipitated by monoclonal antibodies specific for the enzyme. We separated the two proteins and showed that individual antibodies react solely with one or the other. In addition, antibodies to either protein can neutralize enzymatic activity. Our data demonstrate that the proteins are associated in a complex and constitute the subunits of the enzyme.

Herpes simplex virus (HSV) encodes a ribonucleotide reductase whose activity is associated with two proteins of molecular weight 144,000 and 38,000 (144K protein and 38K protein) (1, 9). Genetic studies have shown that a temperature-sensitive mutation in the 144K protein results in a temperature-sensitive reductase, indicating that this protein is essential for enzymatic activity (4). A similar role for the 38K protein is suggested by the homology shared between its coding sequences and those encoding the small subunit of the *Escherichia coli* reductase (13). We have previously reported that monoclonal antibodies able to immunoprecipitate or neutralize enzymatic activity precipitate both viral proteins from crude or purified enzyme preparations (1). Thus, the proteins are either associated in a complex or carry common antigenic determinants (1, 6, 11). To investigate these two possibilities and to establish whether both proteins are necessary for enzymatic activity, we separated them by biochemical or biological means and assayed them for reactivity with monoclonal antibodies and for enzymatic activity.

The monoclonal antibodies used were Bg7, A6, H11, 48S, and 2S (1) and the newly characterized 13αA5, 17αB1, and 17βA4. Two rabbit sera, R1, against the purified enzyme (10), and G, against the amino terminus of the HSV type 1 (HSV-1) 144K protein, were included as controls. All these antibodies immunoprecipitated the 144K and 38K proteins as well as reductase activity from HSV-infected cells, but only Bg7 (1), 13αA5, 17αB1, and 17βA4 (Table 1) neutralized enzymatic activity in solution. With the exception of A6 (HSV-2 specific) and 2S and the G serum (HSV-1 specific), all antibodies are type common although to different degrees (1; this study). Finally, only antibodies A6 and H11 react in immunoblots (6).

As previously reported (10), the 144K and 38K proteins copurify through several steps and cannot be separated even by procedures that dissociate the subunits of bacterial or mammalian reductases (2). Two experimental approaches were followed to obtain preparations containing either one of the proteins of interest. In the first approach, partially purified radiolabeled HSV-2 reductase (8, 10) was preincubated for 1 h in 200 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0)–200 mM 2-mercaptoethanol and chromatographed on Sephacryl S200 in the same buffer (Fig. 1A). Although treatment with the reducing agent did not affect irreversibly the activity of the native enzyme (data not shown), we found that it facilitated separation of the enzyme components by molecular sieving. Column fractions corresponding to molecular weights in the region of 38,000 (peak A) and 144,000 (peak B) were pooled as indicated, concentrated by ammonium sulfate precipitation, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Fractions containing only the 38K component of the reductase complex were obtained by this procedure (Fig. 1B, lane A). However, fractions corresponding to the 144K protein (lane B), although greatly enriched in this species, were contaminated by traces of the 38K protein, suggesting that disruption of the complex or molecular sieving of its components was incomplete.

Each column antigen was assayed for reactivity with antibodies specific for the viral enzyme by immunoprecipitation. The results (Fig. 1B) suggested that each antibody reacts specifically with only one or the other component of the reductase. The 38K protein (antigen A) was precipitated specifically only by H11 and Bg7. The amounts precipitated by the remaining antibodies were not significantly different from that obtained with the control antibody 13αA3 and

TABLE 1. Neutralization of viral ribonucleotide reductase by monoclonal antibodies[a]

Antibody	Immunoglobulin G (μg)	Enzymatic activity (%)	
		HSV-2	HSV-1
13αA5	5	34	2
	10	5	2
	30	2	2
	100	2	2
17αB1	5	64	100
	10	39	92
	30	33	95
	100	20	89
17βA4	100	1	2

[a] Enzyme preparations from HSV-2- or HSV-1-infected BHK-21 cl.13 cells were incubated for 30 min at room temperature with immunoglobulin G purified from the indicated ascitic fluids (1). Reductase activity was then assayed as described previously (1) and expressed as the percentage of the activity measured in the reaction without immunoglobulin G. Control nonimmune immunoglobulin G reduced enzymatic activity only to a minimum of 75% at 100 μg.

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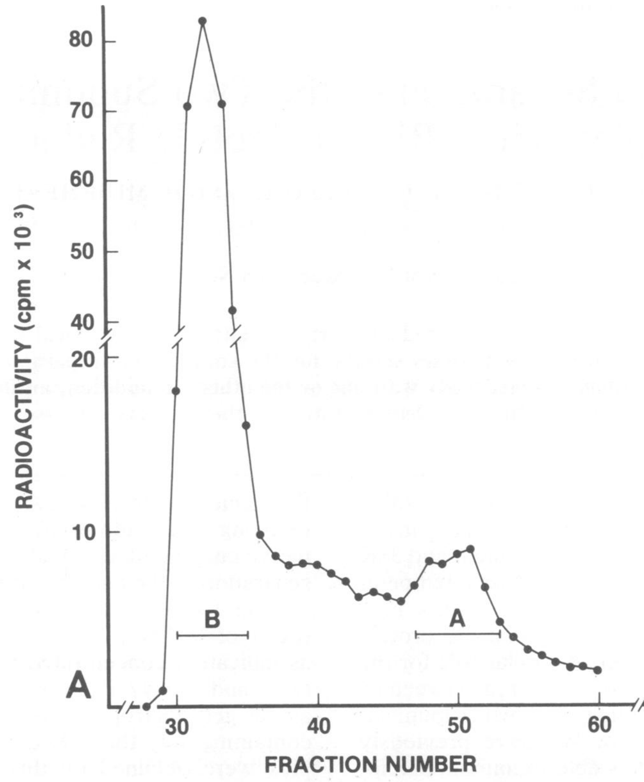
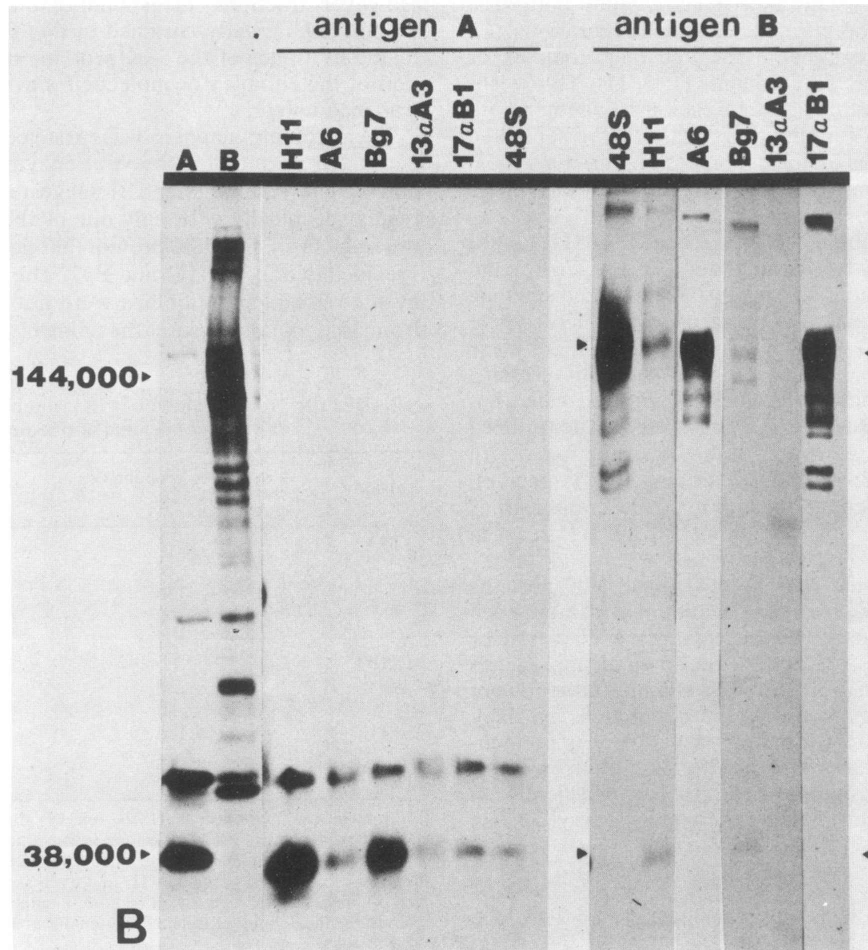

RADIOACTIVITY (cpm x 10 ³)
80
70
60
50
40
20
10
B
A
A
30
40
50
60
FRACTION NUMBER


antigen A
antigen B
A
B
H11
A6
Bg7
13αA3
17αB1
48S
48S
H11
A6
Bg7
13αA3
17αB1
144,000
38,000
B

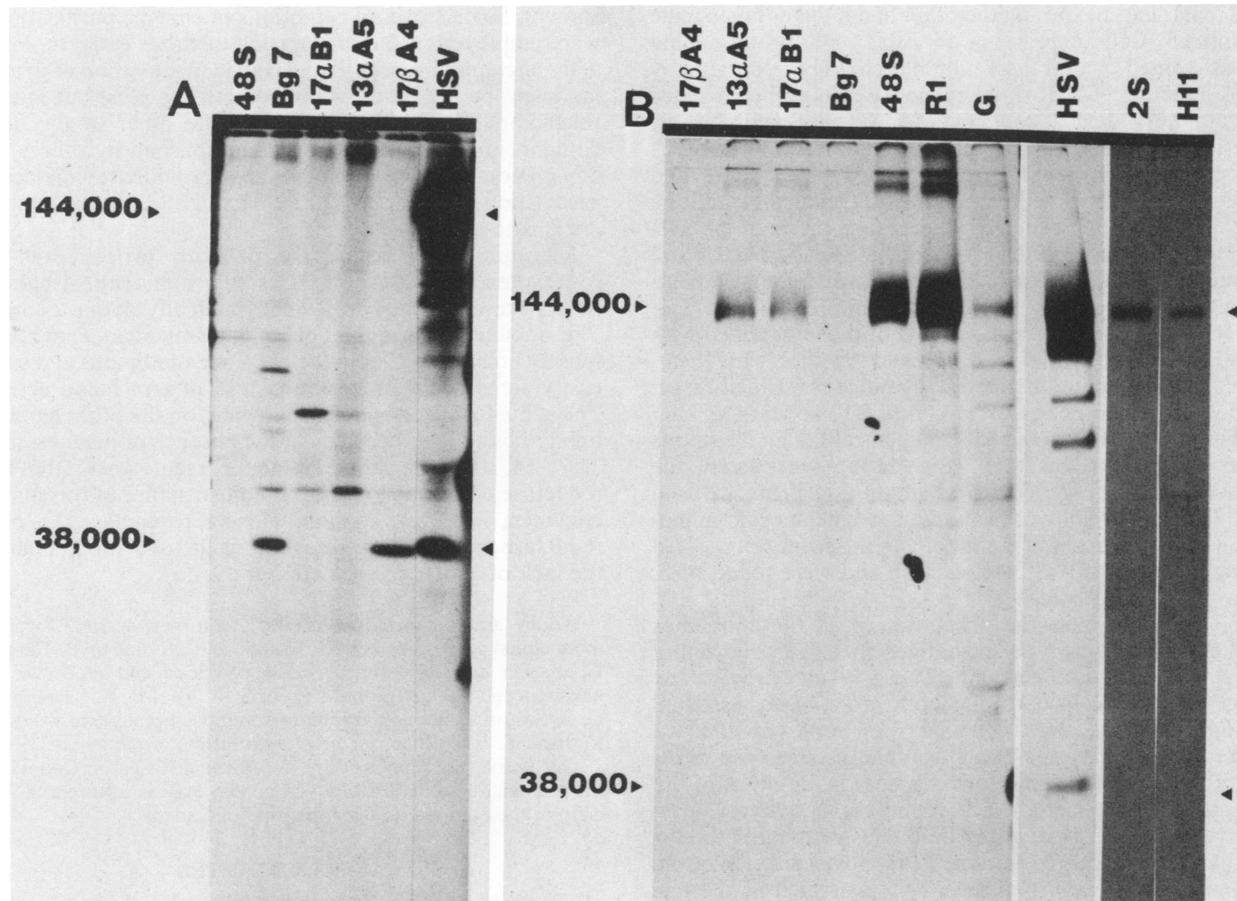


FIG. 2. Reactivity of antireductase antibodies with individual enzyme components from transformed or heat-shocked infected cells. Lysates of labeled cells expressing the HSV-2 38K protein (A) or the HSV-1 144K (ICP6) protein (B) were immunoprecipitated with monoclonal antibodies or with rabbit sera prepared against the HSV-2 enzyme (R1) or against the amino terminus of HSV-1 ICP6 (G). Lanes marked HSV are control immunoprecipitations with R1 serum of lysates of HSV-infected cells expressing both reductase proteins.

were considered nonspecific. Similarly, all immunoprecipitates contained a 45,000-molecular-weight cellular protein (probably actin). Immunoprecipitation of the fraction enriched in the 144K protein (antigen B) indicated that only 48S, A6, and 17αB1 are specific for this protein. Traces of the 38K protein were, however, detectable in these reactions, as in the unreacted antigen (lane B), upon longer exposures. Conversely, antibodies H11 and Bg7, which reacted most efficiently with the residual 38K protein, reacted weakly with the 144K protein. In view of the relative abundance of each protein in antigen B, the weak cross-reactions are likely to reflect the amount of reductase complex still present rather than the true specificity of antibodies toward both proteins.

As a second approach to separating the proteins and to ensure that loss of antibody reactivity was not due to the purification procedure, we produced cells expressing one or the other protein and used them as a source of antigen for immunoprecipitations or enzymatic assays. Cell lines expressing the 38K protein were obtained by cotransfection of mouse Z4 cells (11) with plasmids pSV2neo (12) and p6.2, encoding the gene for the 38K protein (16). After selection with G418 (12), individual cell lines were screened for expression of the 38K protein by immunoprecipitation of radiolabeled cell lysates with antibody Bg7. One of two positive lines was further used to test the remaining antibodies. In addition to Bg7, antibody 17βA4 was capable of reacting with the isolated 38K protein (Fig. 2A). Antibodies

FIG. 1. (A) Separation of the reductase components by gel filtration. Partially purified HSV-2 ribonucleotide reductase was prepared by ammonium sulfate fractionation of crude extracts of infected radiolabeled BHK-21 cl.13 cells (8). The enzyme preparation in 200 mM HEPES (pH 8.0) was preincubated for 1 h in the presence of 200 mM 2-mercaptoethanol, loaded on a Sephacryl S200 superfine column (3 by 93 cm), and eluted with 200 mM HEPES (pH 8.0)–200 mM 2-mercaptoethanol. Column fractions were counted, pooled as indicated, concentrated, and analyzed as in panel B. Only the relevant portion of the column profile is shown here. (B) Reactivity of monoclonal antibodies with individual enzyme components purified by gel filtration. Column fractions pooled as in panel A were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before or after immunoprecipitation with monoclonal antibodies. Lane A and antigen A refer to the pool corresponding to the 38K protein, and lane B and antigen B refer to the pool corresponding to the 144K protein. Monoclonal antibody 13αA3 is a control antibody specific for a 78,000-molecular-weight viral phosphoprotein; all remaining antibodies are specific for the viral enzyme. Molecular weights and arrowheads indicate the relevant proteins.

48S, 17αB1, and 13αA5, on the other hand, did not recognize this antigen. Cells expressing only the 144K protein could not be obtained by cotransfection but could be generated by HSV-1 infection of heat-shocked mouse cells in the presence of cycloheximide (R. H. Persson, C. S. Sartori, J. R. Smiley, and S. Bacchetti, Xth International Herpesvirus Workshop, p. 70, 1985). After removal of the drug and addition of radiolabel and actinomycin D, efficient synthesis of the 144K protein occurred, whereas synthesis of the 38K protein was never detected. Antibodies 13αA5, 17αB1, and 48S reacted with the 144K protein from lysates of heat-shocked infected cells, whereas 17βA4 and Bg7 did not (Fig. 2B). These results are the reciprocal of those obtained with the 38K protein-expressing cells and, together with them, fully confirm the data obtained with antigens purified by gel filtration. On the other hand, antibody H11 appears to react with both the 144K and the 38K antigens although it exhibits greater affinity for the latter (Fig. 1B). As expected, the polyclonal R1 serum reacts with both the 144K (Fig. 2B) and the 38K (data not shown) proteins; the type-specific monoclonal antibody 2S and the antipeptide G serum were tested only against the HSV-1 144K antigen and were found positive.

Table 2 summarizes the properties of all the antibodies tested in terms of serotype and antigen specificity and ability to neutralize the viral enzyme. These results provide the strongest evidence to date that both proteins are essential components of the viral reductase, i.e., represent the two subunits of the enzyme. First, we demonstrated the existence of antibodies specific for only one or the other of the viral proteins associated with reductase activity. Consequently, the reactivity of these antibodies with both proteins in preparations of native enzyme is due to association of the proteins in a complex. Common epitopes, such as the one recognized by H11, appear to be rare and are not generally responsible for the coprecipitation of the proteins. Second, and more relevant to an understanding of the function of each protein, is the fact that neutralization of enzymatic activity occurs with antibodies now known to react specifically with either the 144K (13αA5, 17αB1) or the 38K (17βA4, Bg7) protein. In agreement with this observation is the fact that crude extracts from cells expressing only the 144K or 38K protein were enzymatically inactive (data not shown). Since standard conditions of enzyme purification (8) were employed in the preparation of these extracts, loss of activity cannot be due to nonspecific inactivation of proteins (as might be the case for the column samples) but must be related to the absence of one or the other of the active components of the enzyme. Data published by others after completion of this work have also provided evidence that association of the two proteins might be necessary for enzymatic activity (5).

Ribonucleotide reductase in both procaryotes and eucaryotes generally comprises two nonidentical subunits which are associated in an enzymatically active complex. The subunits of the *E. coli* and mammalian enzyme are loosely bound together in the presence of Mg ions and can be easily separated with resulting loss of enzymatic activity. The subunits of the T4 phage enzyme, on the other hand, are tightly bound together even in the absence of magnesium (2, 3, 7, 14, 15). As shown in the present work, the HSV reductase conforms to the common structure of this class of enzymes; however, it more closely resembles the phage reductase in the tight association of the two subunits and in the lack of allosteric regulation (8, 9, 15).

TABLE 2. Properties of antibodies to the HSV reductase

Antibody	Serotype specificity[a]		Enzyme neutralization[b]		Antigen specificity[c]	
	HSV-2	HSV-1	HSV-2	HSV-1	144K	38K
Bg7	+	+	+	+	−	+
H11	+	+	−	−	+	+
17βA4	+	+	+	+	−	+
13αA5	+	+	+	+	+	−
17αB1	+	+	+	−	+	−
A6	+	−	−	−	+	−
48S	+	+	−	−	+	−
2S	−	+	ND[d]	−	+	ND
R1 serum	+	+	−	−	+	+
G serum	−	+	ND	−	+	ND

[a] Assessed by immunoprecipitation of the reductase components from radiolabeled infected cells.

[b] Measured by the decrease in enzymatic activity upon addition of immune immunoglobulin G.

[c] Determined on the basis of reactivity with the HSV-2 and the HSV-1 144K proteins (ICP10 and ICP6, respectively) or the HSV-2 38K protein.

[d] ND, Not done.

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