Isolation and Partial Chemical Characterization of a 64,000-Dalton Glycoprotein of Human Cytomegalovirus

B. R. CLARK,^{1*} J. A. ZAIA,² L. BALCE-DIRECTO,¹ AND Y.-P. TING²

Division of Immunology, City of Hope Research Institute,¹ and Division of Pediatrics, City of Hope National Medical Center,² Duarte, California 91010

Received 26 May 1983/Accepted 28 September 1983

A guanidinium chloride extract of [³H]glucosamine- and [³⁵S]methionine-labeled virions plus dense bodies of human cytomegalovirus (HCMV) was separated by reverse-phase high-pressure liquid chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluate revealed the major peak to be a glycoprotein with a relative mass of 64,000. This glycoprotein (HCMVgp64) was characterized by amino acid analysis and a high-pressure liquid chromatographic map of its tryptic peptides.

A polypeptide with an M_r (relative mass) of between 64,000 and 69,000 is a major structural protein of human cytomegalovirus (HCMV) (4-9). Immune precipitation of this protein occurs upon reaction of virion components with acute- and convalescence-phase human sera obtained after natural HCMV infection (6). A monoclonal antibody that is specific for this viral product neutralizes virus infectivity and binds to the plasma membrane of HCMV-infected cells (7).

Monolayer cultures of strain 350Q human foreskin fibroblasts (10) were grown in 495-cm² roller bottles and infected on day 7 with HCMV at a multiplicity of infection of 0.1. Roller cultures were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum (DMEM-10) at 37°C, and the extracellular fluid was removed at 96, 144, and 192 h after infection and replaced with fresh DMEM-10. Virions

and dense bodies were collected from extracellular fluid by gradient centrifugation through 20 to 70% sucrose followed by gradient centrifugation through 0 to 40% potassium tartrate-30 to 0% glycerol (1, 9). The band containing virions plus dense bodies (HCMV-db) was collected, washed three times in Tris-buffered saline, suspended in Tris-buffered saline, and frozen at -80°C.

For radiolabeling of virions and dense bodies, roller bottles of cells infected with HCMV were pulsed with either [³⁵S]methionine (New England Nuclear Corp., Boston, Mass.; 1,175 Ci/mmol) or [³H]glucosamine (Amersham Corp., Arlington Heights, Ill.; 29 Ci/mmol) at 5 μ Ci/ml of medium. Labeled HCMV-db was harvested from the extracellular fluid at 96, 144, and 192 h after infection as described above, and at 96 and 144 h the isotope-containing superna-

FIG. 1. HPLC gradient elution profiles of absorbance at 214 nm (—), counts per minute of incorporated [³⁵S]methionine label (.....), or counts per minute of incorporated [³H]glucosamine label (—) obtained for 50 μ l of a 6 M guanidinium chloride extract of HCMV-db radiolabeled with [³⁵S]methionine and [³H]glucosamine. Adsorbed proteins were eluted at room temperature from a Synchropak C-18 reverse-phase column (25 cm by 4.1-mm inside diameter, 300-nm pore size) with a 1.0-ml/min flow rate of the mobile phase (three-step, 60-min gradient of acetonitrile in 0.1% aqueous TFA: 0 to 30%, 0 to 3 min; 30 to 47%, 3 to 39 min; 47 to 70%, 39 to 60 min). The sensitivity of the absorbance measurement was changed from 0.5 to 1.0 absorbance unit full scale at 46 min.

* Corresponding author.

FIG. 2. Photograph of a fixed and Coomassie blue-stained SDS-polyacrylamide slab gel after SDS-polyacrylamide gel electrophoresis (as described in the text) of HPLC eluate fractions collected as shown in Fig. 1. Lane 1 is the electrophoretic separation profile obtained for SDS-solubilized HCMV-db.

tants were filtered through a 0.2- μ m filter and returned to the infected cell cultures.

A frozen suspension containing HCMV-db, isolated by density gradient centrifugation as described above, was thawed and pelleted by centrifugation. The packed pellet was solubilized by suspending it in 6 to 10 volumes of 6 M guanidinium chloride containing 6% β -mercaptoethanol and then boiling the suspension for 12 to 15 min. The cooled solution was centrifuged, and the clear supernatant was filtered and subjected to reverse-phase high-pressure liquid chromatography (HPLC). Aliquots (50 μ l each) of the 6 M guanidinium chloride extract were injected onto a C-18 reverse-phase column (25 cm by 4.1-mm, inside diameter, 30-nm pore, Synchropak RP-P, Synchron, Inc., Linden, Ind.). The adsorbed proteins were eluted at room temperature with a mobile-phase flow rate of 1 ml/min, using a 1-hour, three-step gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA): 0 to 30% over 3 min, 30 to 47% over 36 min, and 47 to 70% over 21 min. Protein in the eluate was detected by monitoring the column effluent at 214 nm with a Waters Associates model 441 absorbance detector and a Hewlett-Packard 3390A integrator-recorder. The eluted peaks were collected manually, vacuum evaporated in a Savant Speed Vac concentrator, and stored over dry silica

gel at -20°C . Figure 1 depicts the gradient elution profiles for absorbance (214 nm) and radioactivity present in an aliquot of a 6 M guanidinium chloride extract of HCMV-db radiolabeled with [^{35}S]methionine and [^3H]glucosamine. The major peak, which elutes at 50 to 52 min (51% acetonitrile), is radiolabeled with both [^{35}S]methionine and [^3H]glucosamine. The minor, later-eluting HPLC peaks have a greater extent of incorporation of [^3H]glucosamine compared with that of [^{35}S]methionine.

Figure 2 shows a Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide slab gel of HCMV-db proteins and also the HPLC eluate fractions (abd'def) indicated in Fig. 1. The essentially pure protein present in fraction d of the HPLC eluate migrates to a band on the gel corresponding to a molecular weight of ca. 64,000 to 66,000. Fraction d', which elutes immediately before the major peak in Fig. 1, contains a single protein with an M_r of 71,000 to 72,000. Fractions e and f contain lesser amounts of the 64,000- to 66,000-molecular-weight protein and other higher-molecular-weight proteins. Figure 3 is a radiofluorogram of an SDS-polyacrylamide slab gel of the HPLC fractions depicted in Fig. 1. The results confirm that the major elution peak is essentially a single protein with a molecular weight of 64,000 to 66,000.

Table 1 contains the data from the amino acid analysis of

FIG. 3. Radiofluorogram of an SDS-polyacrylamide slab gel after electrophoresis of HPLC eluate fractions collected as shown in Fig. 1. Fluorography was performed after impregnation with 2,5-diphenyloxazole as described previously (2).

TABLE 1. Amino acid composition of HPLC-purified HCMVgp64

Amino acid	Mol% ^a
Histidine.....	4.17
Lysine.....	3.97
Tryptophan ^b	
Arginine.....	6.00
Asparagine + aspartic acid.....	8.66
Threonine.....	6.39
Serine.....	6.22
Glutamine + glutamic acid.....	13.0
Proline.....	7.07
Glycine.....	7.49
Alanine.....	7.93
Half-cystine ^b	
Valine.....	7.63
Methionine.....	0.644
Isoleucine.....	3.55
Leucine.....	8.18
Tyrosine.....	3.13
Phenylalanine.....	3.88
Galactosamine.....	2.34 ^c

^a Average of two determinations on 10 µg of HCMVgp64, 48-h hydrolysis.

^b Not determined.

^c Percent by weight.

HPLC-purified HCMVgp64. Protein samples (2 to 10 µg) were hydrolyzed in vacuo at 110°C for 48 h with 6 N HCl containing 0.2% (vol/vol) β-mercaptoethanol. Amino acids were separated on a Beckman 121MB analyzer with a two-column system (3). The results indicate that galactosamine represents ca. 2.3% (wt/wt) of the protein, and the amount of glucosamine recovered was below the level of detection (ca. 0.2 mol%). The protein contains glycine and proline in relatively high amounts. The apparent low content of [³H]glucosamine detected in HCMVgp64 may occur as a result of metabolic conversion of [³H]glucosamine to a derivative of [³H]N-acetylgalactosamine which was then utilized at a low specific activity in protein glycosylation reactions.

The HPLC elution profile of a tryptic digest of HPLC-

purified HCMVgp64 is shown in Fig. 4. Approximately 200 µg of lyophilized, HPLC-purified HCMVgp64 was placed in a 5-ml Pierce Reactivial equipped with a Teflon stirrer. After the addition of 500 µl of 0.2 M NH₄HCO₃, 10 µl of a freshly prepared 0.1-mg/ml solution of trypsin-L-1-tosylamide-2-phenylmethyl chloromethyl ketone (Millipore Corp., Bedford, Mass.) in water was added every 3 h for a total of three additions, and digestion was allowed to proceed at room temperature. After standing overnight, a fourth 10-µl aliquot of freshly prepared trypsin solution was added the following morning. After 3 h, the trypsinized sample was lyophilized in situ. A trypsin digestion without protein prepared under identical conditions served as a control. The lyophilized digest was dissolved in 300 µl of 0.1% (vol/vol) aqueous TFA and transferred to a 1-ml Eppendorf tube along with a subsequent 300-µl wash. The combined solution and wash was centrifuged, and the supernatant was loaded onto a C-18 reverse-phase column (25 cm by 4.6-mm inside diameter, 5-µm particle size, Vydac 218TP54.6 column, Western Analytical Products, Yorba Linda, Calif.).

The HPLC tryptic peptide map of HCMVgp64 is quite reproducible and contains about 40 distinct peaks. Sequence analysis of one of the tryptic peptides has yielded a sequence upon which was based the synthesis of an oligodeoxynucleotide probe for bacterial cloning studies of the viral gene for HCMVgp64.

HPLC is finding increased application for the separation of proteins. It is a particularly powerful tool when used in sequence with other separation methods such as ion-exchange and size exclusion chromatography. Herpesvirus proteins have been purified by a combination of chromatographic methods, but HPLC has not been used preparatively for purification of these polypeptides. This report demonstrates the application of HPLC to the preparative purification of a structural protein of HCMV by which method milligram amounts of HCMVgp64 were obtained.

HPLC purification of preparative amounts of HCMVgp64 has provided abundant material for animal immunizations and immunological assays used in clinical investigations of HCMV disease. Results of immunological studies with rabbit antisera to purified HCMVgp64 will be reported elsewhere.

FIG. 4. HPLC gradient elution profile of 214-nm absorbance (0.5 absorbance unit full scale) for a tryptic digest of 200 µg of HPLC-purified HCMVgp64 (A) and for trypsin alone (B). The lyophilized tryptic digest was dissolved in 300 µl of 0.1% aqueous TFA, injected onto a Vydac 218TP54.6 C-18 reverse-phase column (25 cm by 4.6-mm inside diameter, 5-µm particle size, 300-nm pore size), and eluted at room temperature with a 1-ml/min flow rate of the mobile phase (2-h linear gradient of 0 to 70% acetonitrile in 0.1% aqueous TFA).

This research was supported by Public Health Service grant CA 30206 from the National Cancer Institute, core grant CA 33572 from the National Institutes of Health, and the Harold "Bud" Foley and Charles B. Kaiser, Jr., Research Fund.

We gratefully acknowledge Faye Willman for typing of the manuscript.

LITERATURE CITED

1. Barzilai, R., L. H. Lazarus, and N. Goldblum. 1972. Viscosity-density gradient for purification of foot-and-mouth disease virus. *Arch. Gesamte Virusforsch.* **36**:141-146.
2. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
3. Del Valle, U., and J. E. Shively. 1979. Two-column system for determination of glucosamine, galactosamine, and amino acids on a Beckman 121M amino acid analyzer. Separation of the anomers of glucosamine and galactosamine. *Anal. Biochem.* **96**:77-83.
4. Fiala, M., R. W. Honess, D. C. Heiner, J. W. Heine, Jr., J. Murnane, R. Wallace, and L. B. Guze. 1976. Cytomegalovirus proteins. I. Polypeptides of virions and dense bodies. *J. Virol.* **19**:243-254.
5. Kim, K. S., V. J. Sapienza, R. I. Carp, and H. M. Moon. 1976. Analysis of structural polypeptides of purified human cytomegalovirus. *J. Virol.* **20**:604-611.
6. Pereira, L., M. Hoffman, and N. Cremer. 1982. Electrophoretic analysis of polypeptides immune precipitated from cytomegalovirus-infected cell extracts by human sera. *Infect. Immun.* **36**:933-942.
7. Pereira, L., M. Hoffman, D. Gallo, and N. Cremer. 1982. Monoclonal antibodies to human cytomegalovirus: three surface membrane proteins with unique immunological and electrophoretic properties specify cross-reactive determinants. *Infect. Immun.* **36**:924-932.
8. Sarov, I., and I. Abady. 1975. The morphogenesis of human cytomegalovirus. Isolation and characterization of cytomegalovirus and dense bodies. *Virology* **66**:464-473.
9. Stinski, M. F. 1976. Human cytomegalovirus: glycoproteins associated with virions and dense bodies. *J. Virol.* **19**:594-609.
10. Zaia, J. A., and M. N. Oxman. 1977. Antibody to varicella-zoster virus-induced membrane antigen: immunofluorescence assay using monodisperse glutaraldehyde-fixed target cells. *J. Infect. Dis.* **136**:519-530.