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Identification of the Vaccinia Hemagglutinin Polypeptide from a Cell System Yielding Large Amounts of Extracellular Enveloped Virus

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HeLa, SIRC, and RK-13 cells were compared as to their production of intracellular naked vaccinia virus (INV) and extracellular enveloped vaccinia virus (EEV) after infection with vaccinia strains WR and IHD-J. IHD-J produced more EEV from all three cell lines than did WR, although both strains produced approximately the same quantity of INV. The most efficient EEV release was from RK-13 cells infected with IHD-J, which was 200 times more than from WR-infected SIRC cells. This permitted for the first time the purification of milligram quantities of EEV that contained much fewer cell protein contaminants than could be obtained from HeLa or SIRC cells. The INV surface proteins 200K, 95K, 65K, and 13K were present in both HeLa ahd RK-13 cell-derived INV but were absent in SIRC cell INV. These proteins were absent in EEV from all three cell lines. Four glycoproteins of molecular weights 210×10^3 (210K), 110K, 89K, and 42K and five glycoproteins in the 23K to 20K range plus a nonglycosylated protein of 37K were detected in EEV from the hemagglutinin-positive IHD-J vaccinia strain. The 89K glycoprotein was not present in EEV or membranes from cells infected with the hemagglutinin-negative vaccinia strain IHD-W. Antisera to IHD-W lacking hemagglutinin-inhibiting antibodies did not precipitate the 89K glycoprotein of IHD-J. The only glycoprotein that specifically attached to rooster erythrocytes was the 89K glycoprotein. This evidence indicates that the 89K glycoprotein is the vaccinia hemagglutinin.

During the replication of vaccinia virus, large numbers of infectious virus particles are produced. Most of these particles remain cell associated long after the cell's demise. A small fraction do, however, acquire an envelope and are released (1) as extracellular enveloped vaccinia virus (EEV). The envelope is acquired by the intracellular naked vaccinia virus (INV) either at intracellular membranes or at the cytoplasmic membrane (6). Some data have accrued concerning the structure and function of this envelope. The biological functions of hemagglutination (HA) and the adsorption and penetration of cells are associated with this envelope (13, 14). The envelope has an antigenic composition unrelated to INV (1, 4, 15, 17). The polypeptide structures of INV and EEV grown in HeLa and SIRC cells have recently been compared (12). EEV was found to contain a number of envelope-specific polypeptides. In addition, it was shown that several INV surface proteins were quantitatively altered by the acquisition of an envelope. Further studies of EEV have been severely hampered by the very low EEV yields obtained from HeLa and SIRC cell cultures (12). In this paper we report (i) conditions for virus propagation that yield large quantities of EEV, (ii) some structural properties of EEV, and (iii) the identification of the protein responsible for HA.

MATERIALS AND METHODS

Cells and virus infections. Vaccinia strains IHD-J (HA^+) and IHD-W (HA^-) were provided by S. Dales (Public Health Research Institute, New York, N.Y.), and strain WR (mouse neurotropic) was provided by E. Lycke (Sahlgrenska Hospital, Gothenburg, Sweden). HeLa, SIRC, and RK-13 cells were passaged (1 to 3) every 4 days in Eagle minimal essential medium plus 5% fetal calf serum. Cells for virus production were maintained in minimal essential medium plus 1% fetal calf serum (100 ml/10^8 cells). Cells in stationary culture were infected on day 3 after passage with 1 ml per 10^8 cells (0.1 PFU/cell) with vaccinia strain IHD-J, IHD-W, or WR. When radioactively labeled material was required, cells were infected with 3 PFU/cell, and the radioactive compound was added 4 h postinfection (p.i.). Glycoproteins were labeled by the inclusion of 3 μCi of [^{3}H]glucosamine (New England Nuclear Corp.) per ml in medium with 10% the usual

glucose concentration. Proteins were labeled with 3 μCi of [^{35}S]methionine (Amersham) per ml in medium containing one-eighth the usual amount of methionine. Virus infectivity was determined by plaque titration on A-549 cells (13).

Virus purification. Unlabeled material was collected at 48 h p.i., and radioactively labeled material was harvested at 24 h p.i. The medium was clarified at 5,000 rpm for 5 min in a Sorvall GSA angle rotor, and then the EEV was sedimented for 30 min at 10,000 rpm. The EEV sediment was suspended in phosphate-buffered saline (PBS). INV was obtained from PBS-washed cells by first swelling the cells in distilled water (1 ml/10^8 cells) for 10 min and then rupturing them with 10 strokes of a Dounce homogenizer. The large debris and nuclei were sedimented at 3,000 rpm for 10 min. The EEV and INV preparations were both treated with a Rapidis (Ultrasonic Ltd.) sonifier at 30 μM for 10 s before purification.

INV and EEV were purified by two consecutive equilibrium cesium chloride centrifugations. Virus material (3 ml) was centrifuged for 60 min at 30,000 rpm in an SW40 rotor in a gradient formed by prelayering 1.30-g/ml (2 ml), 1.25-g/ml (3 ml), and 1.20-g/ml (4 ml) cesium chloride solutions. The INV and EEV virus banded at 1.27 and 1.23 g/ml, respectively. A light-diffracting material above the EEV band was carefully removed with a Pasteur pipette. The INV and EEV bands were than collected dropwise from the bottom of the tube. The virus suspensions were diluted 1:3 with PBS and recentrifuged for 60 min at 30,000 rpm in cesium chloride gradients similar to the first centrifugation. The protein content of purified virus suspensions was measured by UV spectrophotometry (1 unit of optical density at 260 nm = 64 μg) (7).

Purification of cell membranes. RK-13 cells infected with 3 PFU of IHD-J or IHD-W per cell and labeled with [^{3}H]glucosamine starting at 4 h p.i. were harvested at 24 h p.i. Cell membranes were purified by a modification of published methods (2). The cells were washed once in PBS and then suspended in distilled water (3 ml/10^8 cells) and allowed to swell for 10 min before disruption with 15 strokes of a Dounce homogenizer. The nuclei were removed by centrifugation three times at 1,000 rpm for 1 min. The supernatant was then centrifuged for 1 h at 30,000 rpm in an SW40 rotor on discontinuous sucrose gradients (2 ml of 60%, 3 ml of 38%, and 4 ml of 10%). The band at the 10–38% sucrose interface was collected and frozen.

Antisera and immunoprecipitation. Rabbit antisera to live IHD-W and live IHD-J were raised as previously described (14). Antisera (0.1 ml) were incubated for 2 h at 22°C with [^{3}H]glucosamine-labeled IHD-J-infected RK-13 cells (ca. 5×10^7 cells in 1 ml of PBS). The cells were then sedimented and washed once with PBS. The cells were subsequently disrupted, and the nuclei were removed as described for membrane purification. The supernatant was centrifuged at 7,000 rpm for 30 min, and the sediment was dispersed by sonication at 4°C in 1 ml of a modified (10) RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.01 M Tris-hydrochloride, pH 7.4, and 100 K units of Tyasylol Aprotinin per ml). Unsolubilized material was sedimented at 7,000 rpm for 30 min. Immune complexes were precipitated by addition of 100 μl of a 1:1 slurry of *Staphylococcus aureus* protein A bound to Sepharose CL4B (Pharmacia, Sweden) in 0.01 M phosphate buffer (pH 7.2) at 4°C for 1 h. This use of protein A bound to Sepharose is a modification of its first application in affinity chromatography for the isolation of antigen-antibody complexes (11).

PAGE. Sample preparation and conditions of polyacrylamide gel electrophoresis (PAGE) in a slab gel apparatus (16) with a modified Laemmli discontinuous sodium dodecyl sulfate buffer system (9) were as previously described (12). The stacking and separation gels were 4.5% and 15% acrylamide, respectively, with *N,N'*-ethylenebisacrylamide concentrations of 0.12% (stacking) and 0.18% (separation). The stacking gel also contained 0.6% agarose. Samples of 100 μg of virus were analyzed in 5-mm channels, and gels were protein stained with Coomassie brilliant blue. Radioactively labeled proteins were detected by scintillation autofluorography (3).

RESULTS

Comparison of the yield of EEV from different cell lines. Table 1 shows a comparison of the WR and IHD-J vaccinia strains grown in SIRC, HeLa, and RK-13 cell cultures. No significant difference was observed in the amount of INV produced by the two vaccinia strains in the three cell lines. The yield of EEV, however, showed a considerable dependence on virus strain and cell line. The IHD-J strain always released more EEV than strain WR. The WR strain released less EEV from SIRC cells than from HeLa or RK-13 cells, and the IHD-J strain released considerably more EEV from RK-13 cells than from SIRC of HeLa cells. The IHD-J strain yielded approximately 200 times more virus from RK-13 cells than WR strain from SIRC cells. Twenty-seven percent of infectious virus produced during IHD-J vaccinia replication in RK-13 cells was released as EEV.

Optimal EEV yields were obtained from RK-

TABLE 1. *EEV production as a function of virus strain and cell line*[a]

Virus strain	Cell line	Total EEV (PFU × 10^8)	EEV increase compared to WR-SIRC	Total INV (PFU × 10^9)	EEV/(EEV + INV) (%)
WR	SIRC	0.14	—	8.5	0.16
WR	HeLa	0.63	4.5	8.5	0.74
WR	RK-13	0.52	3.7	9.5	0.54
IHD-J	SIRC	9.0	64	9.8	8.4
IHD-J	HeLa	7.0	50	9.2	7.1
IHD-J	RK-13	28	200	9.2	27

[a] Cell cultures were infected as described in the text. At 48 h p.i. the cells and medium were separated. INV was released by Dounce homogenization and sonication. INV and EEV were plaque assayed in triplicate as described in the text.

13 cells that were cultivated and maintained in medium containing fetal calf serum instead of calf serum. Virus release was greater at 37°C than at 33°C but was not dependent on multiplicity of infection between 1.0 and 0.01 PFU/cell. Between 25 and 40% of the virus produced was released as extracellular virus. When the EEV was purified from 30 Roux bottles on CsCl gradients, the virus yield varied from 4 to 8 mg. The quantity of virus banding in CsCl at an INV density was always less than 5% of the total extracellular virus.

The production of INV and EEV from RK-13 cells was monitored during a one-step growth cycle (Fig. 1). Newly replicated INV was first apparent between 4 and 6 h p.i. and increased until 12 h p.i. EEV was also detectable at 6 h p.i. but increased until 16 h p.i. Although INV was first detectable at 6 h, the actual latent time was probably less. The high levels of uneclipsed infecting virus made a more exact determination of the latent time difficult. Similarly, the lag time between the appearance of INV and its release as EEV remains undetermined. The most active period for the generation of both virus populations was between 6 and 12 h p.i., when INV increased by approximately 40-fold and EEV by 200-fold. At the end of the replication cycle, 25% of the total virus was released as EEV.

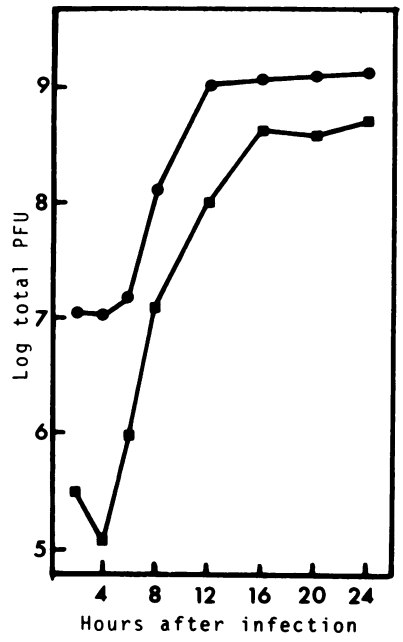

FIG. 1. *RK-13 cells in 25-cm² plastic bottles were infected with IHD-J vaccinia at a multiplicity of infection of 2 PFU/cell. After 60 min at 37°C, the unattached virus was removed by three washes with medium and then incubated at 37°C with 10 ml of medium per flask. At the indicated times the cells and medium were separated by centrifugation. EEV (■) in the supernatant and INV (●) from the sonically disrupted cells were assayed by plaque assay with four petri dishes per material. Time 0 is taken as the time at which virus was added to the bottles.*

EEV and INV proteins from HeLa-, SIRC-, and RK-13-cultivated vaccinia virus. The very high EEV yields from RK-13 cells greatly facilitate structural studies. It was therefore particularly appropriate to compare the relative purity of EEV grown in RK-13 cells with EEV obtained from HeLa and SIRC (Fig. 2).

The major polypeptides are very similar in both distribution and concentration. Nevertheless, there are considerable differences visible in certain less prominent polypeptides. HeLa cell-derived EEV contains a number of minor polypeptides in the upper third of the gel that are not present in RK-13 cell-cultivated EEV. These extra proteins are presumably cell or serum contaminants. SIRC cell-cultured EEV also shows fewer such contaminants than HeLa cell-grown EEV, in agreement with previous results (12). The most conspicuous difference is the presence of a polypeptide in HeLa cell EEV that comigrates with cellular actin.

We have previously shown (12) that certain INV surface proteins can vary in their concentration as a function of the cell line used for cultivation. Figure 3 extends these observations to INV grown in RK-13 cells. Most obvious is that proteins 200K, 95K, 65K, and 13K, which are absent in SIRC cell INV, are present in HeLa cell- and RK-13 cell-derived INV. It is notable, however, that the 200K protein was always reduced in RK-13 INV compared to HeLa INV. It is also apparent in Fig. 3 that RK-13 EEV lacks these four proteins, as does EEV from HeLa and SIRC cells (see Fig. 2 and reference 12). No differences were seen in the 30K and 27K polypeptides in RK-13 INV compared to EEV. One polypeptide at 37K present in RK-13 EEV but absent from INV is an envelope-specific protein that is also present in HeLa and SIRC cell EEV (see Fig. 2 and reference 12).

Glycoproteins of EEV. The glycoprotein composition of EEV was analyzed by PAGE of [^{3}H]glucosamine-labeled virus grown in Eagle minimal essential medium containing fructose instead of glucose. The substitution of fructose for glucose resulted in a fivefold increase of [^{3}H]glucosamine incorporation into EEV. [^{3}H]-fucose was previously used (12), but the use of [^{3}H]glucosamine in a fructose minimal essential medium resulted in 10 to 20 times more radiolabel incorporation than could be achieved with

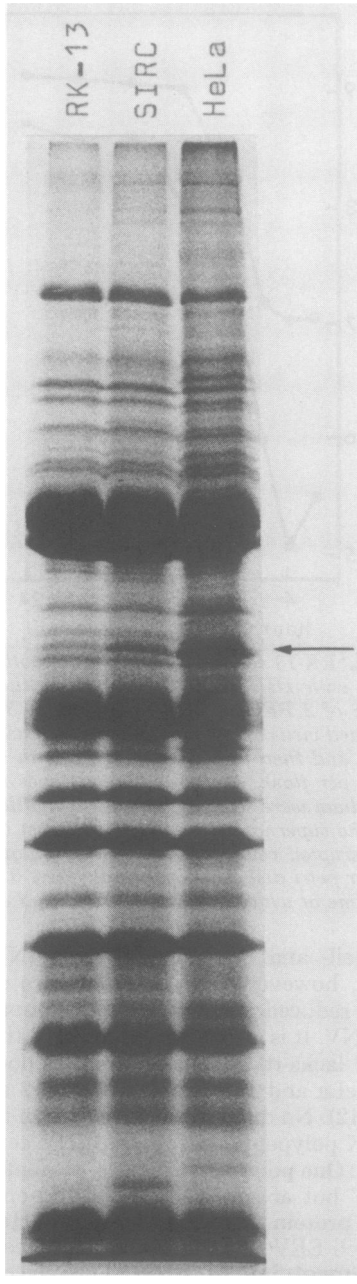

FIG. 2. *PAGE comparison of EEV from HeLa, SIRC, and RK-13 cells twice CsCl purified. The arrow denotes the position of cellular actin.*

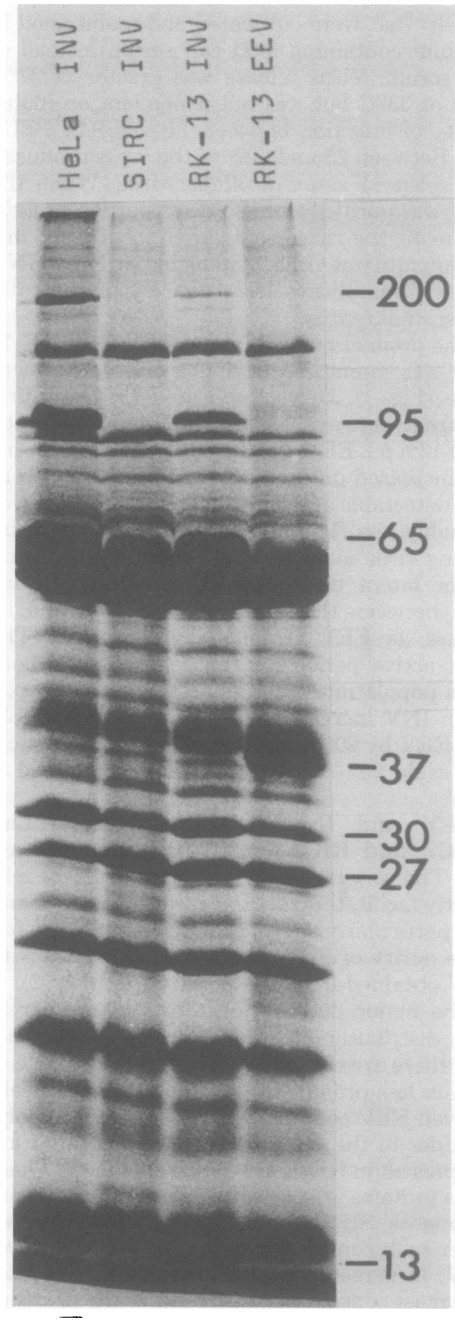

FIG. 3. *PAGE analysis of INV from HeLa, SIRC, and RK-13 cells compared to EEV from RK-13.*

[^{3}H]fucose. Figure 4 shows a scintillation autofluorogram of [^{3}H]glucosamine- and [^{35}S]methionine-labeled EEV and also [^{3}H]glucosamine-labeled INV. One INV protein is glycosylated, in agreement with previous findings (12). All of the major INV proteins show a low level of [^{3}H]glucosamine incorporation. This is probably a consequence of the [^{3}H]glucosamine being metabolized to amino acids and a subsequent incorporation into viral proteins. The EEV-spe-

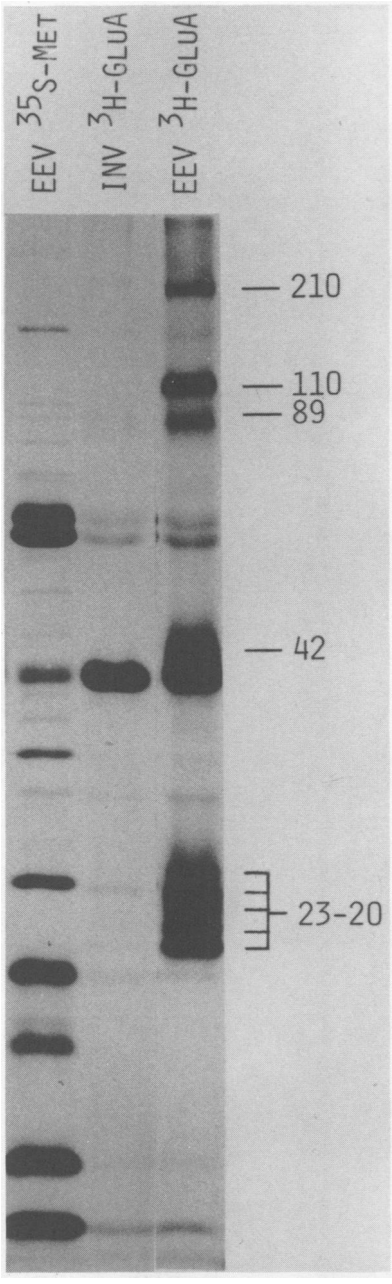

FIG. 4. *PAGE analysis of EEV and INV labeled with [^{3}H]glucosamine, and [^{35}S]methionine-labeled EEV from RK-13 cells.*

cific glycoproteins have molecular weights of 210 $\times 10^3$ (210K), 110K, 89K, and 42K, and a complex of five glycoproteins has molecular weights between 23K and 20K. The EEV-specific 37K protein that comigrates with the INV glycoprotein was shown in a separate experiment to be nonglycosylated, in agreement with previous results (12). The nine glycoproteins and one nonglycosylated protein of EEV were not detectable in EEV from RK-13 cells that were [^{35}S]methionine labeled prior to virus infection.

PAGE analysis of EEV and cell membranes from HA$^+$ and HA$^-$ vaccinia strain-infected cells. The IHD-J vaccinia strain produces a hemagglutinin during infection, whereas the genetic variant, IHD-W, does not (8). We have taken advantage of this biological difference to ascertain if there exists any relationship of the hemagglutinin activity to the glycoprotein composition of EEV and cell membranes derived from cells infected with these strains. Figure 5 shows the glycoproteins found in cell membranes from IHD-J- and IHD-W-infected RK-13 cells compared to the glycoproteins present in EEV-J. It is apparent that the EEV-J glycoproteins at 42K and 23-20K comigrate with glycoproteins in cell membranes from both IHD-J- and IHD-W-infected cells. It is also evident that the EEV-J 89K glycoprotein is the dominant glycoprotein in IHD-J-infected cell membranes, whereas it is absent from IHD-W-infected cell membranes. The 110K glycoprotein could not be discerned in IHD-J-infected cell membranes due to the predominance of the 89K glycoprotein; however, it was apparent in IHD-W-infected cell membranes. Figure 6 shows a scintillation autofluorogram of [^{3}H]glucosamine-labeled EEV-J and EEV-W. The salient difference in the glycoprotein composition was the near-complete absence from EEV-W of the 89K glycoprotein. The same result was obtained using [^{3}H]fucose for glycoprotein labeling. A similar comparison (gel not shown) of the envelope proteins of EEV-J and EEV-W revealed that there was no difference in the Coomassie blue staining pattern in the 89K protein region.

Precipitation of IHD-J-infected cell membrane proteins with HI$^+$ and HI$^-$ antisera. Rabbit antiserum raised against live IHD-W does not contain hemagglutinin-inhibiting (HI) antibodies, whereas rabbit antiserum to live IHD-J has high HI antibody titers. Figure 7 shows the glycoproteins from IHD-J-infected cell membranes that were precipitated by these two antisera. Sera containing HI antibodies precipitated the 89K glycoprotein and several glycoproteins that comigrate with EEV proteins. By contrast, antisera lacking HI antibodies precipitated several glycoproteins that comigrate with EEV glycoproteins but did not precipitate the 89K glycoprotein.

Adsorption and elution of the vaccinia hemagglutinin from rooster erythrocytes.

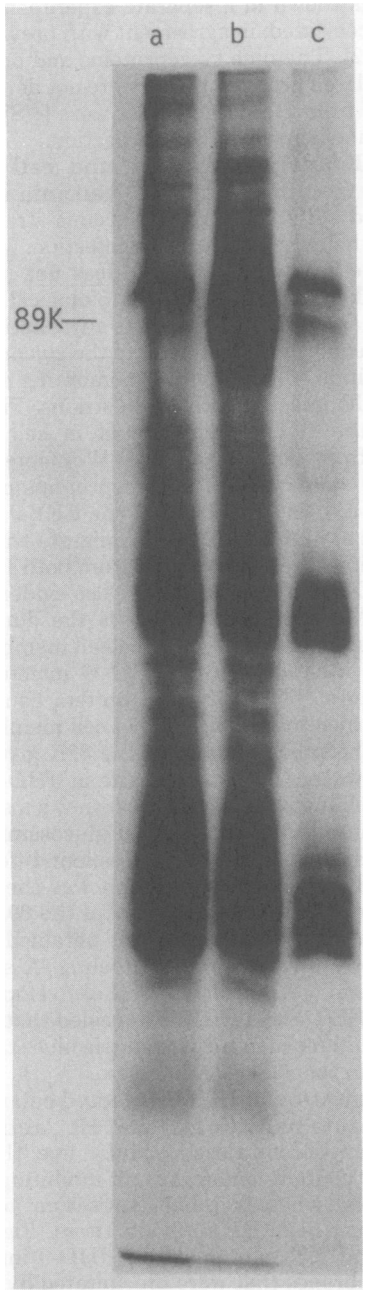

FIG. 5. *Comparative analysis of equal amounts of [^{3}H]glucosamine-labeled cell membranes from cells infected with IHD-W (a) or IHD-J (b) vaccinia virus. EEV-J (c) labeled with [^{3}H]glucosamine is included as a marker.*

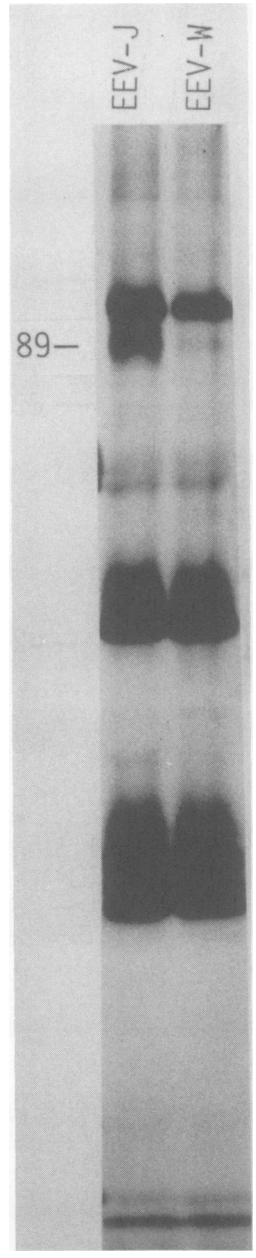

FIG. 6. *Comparison by PAGE of [^{3}H]glucosamine-labeled EEV from IHD-J (HA$^+$) and IHD-W (HA$^-$) vaccinia strains.*

The preceding data provide circumstantial evidence in favor of the 89K glycoprotein being the vaccinia hemagglutinin. A more direct approach was therefore required. Vaccinia hemagglutinin-sensitive rooster erythrocytes (25% concentration) were treated with 0.1% glutaraldehyde in PBS for 30 min at 4°C and then washed three times with PBS. This treatment largely prevented erythrocyte lysis during subsequent

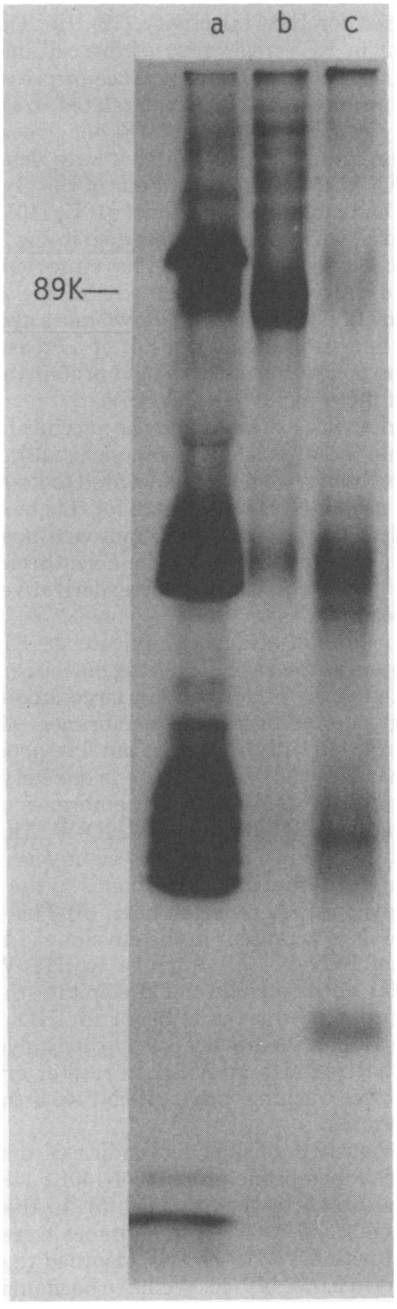

FIG. 7. *[^{3}H]glucosamine-labeled IHD-J-infected RK-13 cells were mixed with either antiserum to IHD-J (HI = 1,024) (b) or antiserum to IHD-W (HI < 2) (c), and the immune complexes were precipitated as described in the text. Equal amounts of radioactive material were then subjected to PAGE. EEV-J (a) labeled with [^{3}H]glucosamine is included as a marker.*

washing and elution steps. [^{3}H]glucosamine-labeled cell membranes from IHD-J-infected RK-13 cells were adsorbed three times with 1% treated rooster erythrocytes for 1 h at 37°C with constant agitation. The pooled erythrocyte sediments were twice washed with 0.01% Nonidet P-40 (NP-40) in PBS. This released about 90% of the radioactive label from the erythrocytes. If the erythrocytes were washed with 0.05% NP-40 instead of 0.01% NP-40, no radioactivity remained associated with the erythrocytes. The residual radioactive label was then eluted with 1% NP-40 in PBS and ethanol precipitated. Figure 8 shows the result of one such adsorption and elution experiment. The only glycoprotein that remained attached to the rooster erythrocytes after the 0.01% NP-40 washes and that could subsequently be eluted by 1% NP-40 was the 89K glycoprotein.

DISCUSSION

Structural-functional studies of virus particles are greatly facilitated by the availability of large quantities of purified virus. Only very low yields of EEV have been previously obtained (1, 4, 12–15, 17). In the present paper we report conditions of vaccinia cultivation that dramatically increase EEV yields. EEV release was maximal from RK-13 cells infected with the IHD-J vaccinia strain. This virus-cell combination releases between 25 and 40% of its virus as EEV or approximately 30 to 80 EEV infectious particles per cell. This yield has permitted us for the first time to purify milligram quantities of EEV from only 30 Roux bottles (ca. 3×10^9 cells). It is, however, not only the higher EEV yields from RK-13 cells that make this cell line more useful than the more commonly used HeLa cells. The amount of contaminating cellular material remaining in RK-13-derived EEV after two CsCl purification steps is significantly less than is found in HeLa-derived EEV. Two CsCl gradient centrifugations avoided virus particle aggregation that was observed in consecutive sucrose and CsCl centrifugations used previously for EEV purification (12). The combined effect of high yield and purity of EEV produced from IHD-J-infected RK-13 cells makes this the most suitable source of EEV thus far reported.

The diversity in the INV polypeptide patterns observed for IHD-J vaccinia grown in HeLa and SIRC cells (12) was confirmed here. The absence of the 200K, 95K, 65K, and 13K proteins in SIRC cell- compared to HeLa cell-derived INV remains unexplained. The fact that RK-13 INV more closely resembled HeLa INV (although the 200K protein was significantly reduced) than

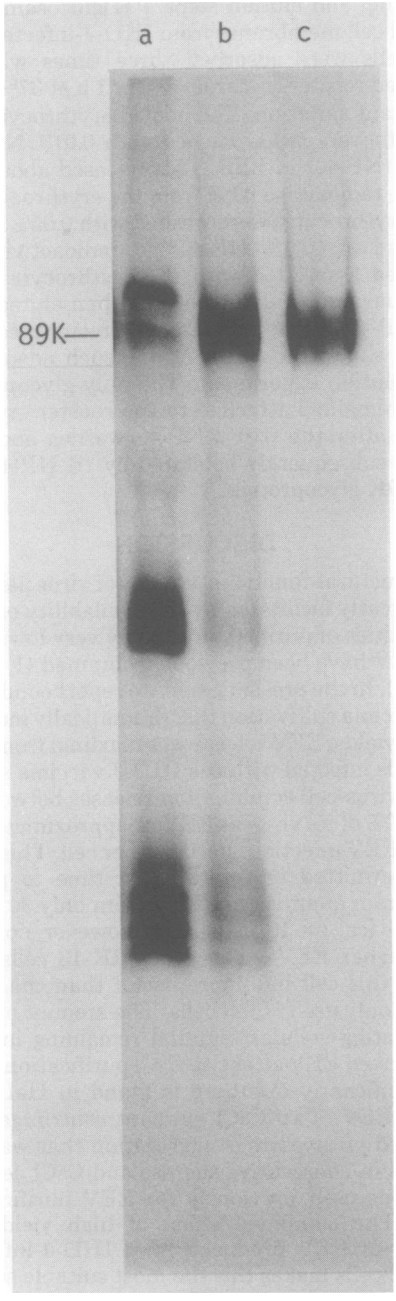

FIG. 8. *[^{3}H]glucosamine-labeled IHD-J-infected cell membrane proteins were adsorbed and eluted from rooster erythrocytes as described in the text. The eluted material (c) was compared by PAGE with the original membrane preparation (b) and [^{3}H]glucosamine-labeled EEV-J (a).*

SIRC INV is also unexplained. Nevertheless, the available evidence does permit the statement of three points concerning these INV proteins. (i) These proteins are surface proteins that are not required for INV infectivity (12). (ii) They are absent in EEV regardless of the cell line used for cultivation. (iii) The absence or presence of these proteins in INV is not related to the efficiency of EEV release. A 37K nonglycosylated protein and nine glycoproteins were detectable in EEV from RK-13 cells. Four of the glycoproteins had molecular weights of 210K, 110K, 89K, and 42K, while five were observed in the 23-20K molecular weight range. We have previously reported only three glycoproteins in the 23-20K region (12). The detection of two more glycoproteins here is probably a result of increased resolution of low-molecular-weight proteins and the use of more highly labeled EEV.

Earlier work showed that the vaccinia hemagglutinin is an envelope component (13). In the present study, we have endeavored to determine what protein(s) is responsible for this biological activity. The experimental approach made use of the fact that IHD-J vaccinia synthesizes hemagglutinin whereas its genetic derivative IHD-W does not.

Four lines of evidence implicate the 89K glycoprotein as the vaccinia hemagglutinin. (i) Cells infected with IHD-J contain large amounts of this glycoprotein in their membranes, whereas cells infected with IHD-W do not. Previous work (18) has also shown that there is one very dominant glycoprotein found in membranes of cells infected with IHD-J but not IHD-W. Although the glycoprotein's molecular weight was not reported, it presumably corresponds to the major 89K glycoprotein reported here. (ii) This glycoprotein is also present in the envelope of EEV-J but not EEV-W. (iii) Antisera to IHD-W that lack HI antibodies do not precipitate the 89K glycoprotein, whereas antisera to IHD-J containing HI antibodies do. (iv) The only glycoprotein that remains attached to rooster erythrocytes after washing with 0.01% NP-40 is the 89K glycoprotein.

Previously published data indicated that vaccinia cell membrane proteins of 150K, 34K, and 12K adsorbed to erythrocytes (5). In these experiments, infected cell membranes were solubilized with 0.5% NP-40 and adsorbed to erythrocytes in 0.05% NP-40. We have been unable to reproduce these experiments. Indeed, our own experiments show that no proteins adsorb to erythrocytes in the presence of 0.05% NP-40.

It is particularly noteworthy that the nonglycosylated form of the 89K protein seems to be present in both EEV (reported here) and cell membranes (18) from cells infected with the HA$^-$ IHD-W vaccinia strain. Tryptic peptide analysis of this protein from IHD-W- and IHD-J-infected cell membranes revealed that the two proteins are very similar (preliminary data re-

ported in reference 19). One would therefore expect that antisera to the IHD-W strain would precipitate the very similar protein from IHD-J-infected cells. This was not, however, observed in our precipitation experiments. These two sets of data may be explained by assuming that glycosylation of the 89K protein causes a marked conformational change in the protein's secondary structure such that antibodies to IHD-W no longer can recognize the glycosylated 89K protein. Glycosylation may also have a role in the biological activity of HA. It has been shown that, when glycosylation is inhibited in cells infected with the HA^+ IHD-J vaccinia strain, the presence of a nonglycosylated 89K protein was correlated to a drastic reduction in HA activity (19). Thus, glycosyl residues may themselves directly cause HA, or the glycosylation-related protein rearrangement may expose new sites on the 89K protein that are responsible for HA.

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