Virus-Receptor Interaction in the Adenovirus System

I. Identification of Virion Attachment Proteins of the HeLa Cell Plasma Membrane

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Received 17 September 1980/Accepted 5 January 1981

Plasma membranes from HeLa cells were isolated in a two-phase polymer system. To compare the efficiency of attachment protein extraction, a normalized assay for the assessment of adenovirus type 2 (Ad2) receptor-active components interfering with the attachment of Ad2 to HeLa cells was developed. An optimized detergent extraction procedure, 0.5% Triton X-100, was used, and solubilized membrane proteins were radioisotope labeled in vitro. Proteins with affinity for Ad2 virions were quantified and identified in a sucrose gradient sedimentation assay and by affinity chromatography with cross-linked Ad2 virions immobilized to AH-Sepharose 4B. From virions recovered in the sucrose gradient system, one major membrane component of high affinity was identified with a polypeptide molecular weight of around 40,000. Glycosylated proteins isolated by wheat germ lectin chromatography with high affinity for immobilized virus particles were isolated, and two major components with apparent molecular weights of 40,000 and 42,000 were identified. We suggest that a glycosylated protein with high affinity for Ad2 virions and a polypeptide molecular weight of 40,000 to 42,000 is one component of the Ad2 attachment site on HeLa cells.

All virus infections start with the viruses recognizing components on the surface of the cell that is about to be infected. The extracellular interactions between animal viruses and cells have been extensively reviewed and described by Lonberg-Holm and Philipson (27). More recently, Kohn has elaborated on a selected number of viruses interacting with cellular membranes (24).

The cellular surface components of interest for the infecting virions have usually been referred to as viral receptors. A more appropriate term, virus attachment proteins, has been suggested (32); this term implies that the cells do not synthesize membrane components for the a priori function of being the structures responsible for reception of future viral infections.

In the adenovirus system, the virion part of the virus–cell-recognizing system is the well-characterized fiber antigen (10, 34, 37) located at the 12 vertices of the virion (38). Previous studies also indicate that plasma membrane components that are protein in nature, and possibly glycosylated, are important in the initial steps of virus-cell interaction (31, 34). Attempts have been made to isolate and characterize the components of the attachment site for adenovirus type 2 (Ad2) on KB cells, using highly purified fiber and penton structures. Hennache and Boullanger (18) identified three major Ad2–fiber-recognition polypeptides with apparent molecular weights of 78,000, 42,000, and 34,000.

With an assay designed to measure and normalize adenovirus attachment protein activity of HeLa cell plasma membrane extracts, we tried to optimize the conditions for preparation of virus recognition proteins. Throughout efforts to identify and isolate these components, i.e., by sucrose gradient sedimentation analyses and affinity chromatography techniques, complete virus particles were used to mimic the complex situation in vivo.

MATERIALS AND METHODS

Cells. HeLa cells were maintained in suspension cultures at cell densities of 2 × 10⁶ to 6 × 10⁶ cells/ml in Eagle minimal essential medium (EMEM) supplemented with 8% fetal calf serum and gentamicin (5 μg/ml).

Infections and virus purification. HeLa cells grown in spinner cultures were synchronously infected with Ad2 at a multiplicity of 2,000 particles per cell as previously described (13). For radioisotope labeling, virus was propagated in the same way. [3H]thymidine (56 Ci/mmol; 25 μCi/ml) was added 10 h postinfection and maintained until harvest of cells at around 40 h postinfection. Virus was purified and stored as described elsewhere (12).

Plasma membrane isolation. HeLa cell plasma membranes were isolated from batches of 5 × 10⁶ cells in a discontinuous sucrose gradient system as described by Bosmann et al. (5), modified with a cushion
of 60% (wt/vol) sucrose applied at the bottom of the centrifuge tubes. Five fractions were collected from each tube and denoted as follows: fraction I, the cushion of 60% sucrose; fraction II, the density region corresponding to the interphase between the sucrose concentrations of 45 and 35%, including the entire portion of 45% sucrose; fraction III, the interphase region between the sucrose concentrations of 35 and 25%; fraction IV, the interphase region between concentrations of 25 and 20%; and fraction V, the remaining uppermost portion.

Plasma membranes were also prepared in a two-phase polymer system consisting of polyethylene glycol 6000 and dextran T-500 as described by Brunette and Till (7) and modified by Jay et al. (22). Batches up to 2 x 10^7 cells were processed, and 5 x 10^6 cells per 120 ml of mixed-phase system were used. At the end of the separation, the interphase was collected and diluted five times in 50 mM Tris-hydrochloride buffer, pH 7.3. The membranes were sedimented at 4,500 x g for 15 min at 4°C, washed once, and resuspended in the buffer above to yield a protein concentration of 4 to 8 mg/ml.

**Enzyme marker assays.** 5'-Nucleotidase was assayed by the method of Heppel and Hilmo (19) as modified by Johnsen et al. (23). Na+, K+-ATPase was determined by the method of Wallach and Kamat (39). Phosphate determinations were in both cases done according to the method of Chen et al. (9). Reduced nicotinamide adenine dinucleotide phosphate cytochrome c reductase was assayed by the method of Bricard and O'Brien (30). Protein was determined by the method of Hartree (16), with bovine serum albumin (BSA) as the standard.

**Solubilization of membrane proteins.** All solubilization experiments were carried out on plasma membranes isolated by the two-phase method.

(i) **High-salt procedure.** Solid KCl (36) was added to a final concentration of 3 M; the samples were then shaken until the salt dissolved and further incubated for 16 to 20 h at 8°C. The samples were subsequently centrifuged at 100,000 x g for 1 h at 4°C, and the supernatants were dialyzed against 50 mM Tris-hydrochloride buffer, pH 7.3.

(ii) **Organic solvent procedures.** Butanol (29), pentanol (40), and pyridine (3) were separately applied as extracting agents, each at a final concentration of 10% (vol/vol). After incubation for 1 h at 8°C with shaking every 15 min, the samples were centrifuged and dialyzed as described for the high-salt procedure.

(iii) **Detergent procedures.** Various concentrations of the following detergents were used: Triton X-100 [polyoxyethylene(9,10)-p-t-octyl-phenol], Tween 20 (polyoxyethylene sorbitan monolaurate), and sodium deoxycholate (DOC) (17). After detergent treatment, the samples were incubated for 1 h at 8°C with shaking every 15 min and finally centrifuged as above. The dialysis step was omitted.

In vitro labeling of proteins. Triton X-100-solubilized proteins were labeled for 2 h at 26°C by reductive alkylation, using sodium cyanoborohydride and [3H]formaldehyde as described by Dottavo-Martin and Ravel (11). The reaction mixtures were passed through PD-10 columns with Sephadex G-25M equilibrated and eluted with 40 mM sodium phosphate buffer, pH 7.0, containing 0.037% Triton X-100. The labeled proteins were recovered in the void volume and monitored by assessment of trichloroacetic acid-precipitable radioactivity. The specific radioactivity of labeled proteins ranged from 2.7 x 10^6 to 3.0 x 10^9 cpm per mg of protein.

Labeling of membrane proteins with the Bolton-Hunter reagent was performed essentially as described by the manufacturer. After labeling, the samples were separated from unincorporated isotope by passing through a PD-10 column with 0.15 M NaCl-0.037% Triton X-100 in 0.015 M sodium phosphate buffer, pH 7.3, as the eluant. Labeled material was detected by radioactivity measurements without prior precipitation. Labeling efficiencies were in the range of 90%, and the specific activities of labeled material were about 9 x 10^9 cpm/mg of protein.

**Assay of receptor-active material.** The principle of the assay was based on the assumption that solubilized plasma-membrane proteins compete with viruses during the course of virus-cell interaction and thus inhibit virus attachment to cells.

(i) **Incubation 1.** Five microliters of highly purified [3H]thymidine-labeled Ad2 virions (8.5 x 10^9 particles; 69,400 cpm) was mixed with 120 µl of double-distilled water (for the control series) or with 120 µl of protein sample (for the sample series) and 125 µl of 2X EMEM designed for suspension cultures without serum, which was omitted in all subsequent steps. To ensure proper solubility of all reagents, Triton X-100 was added at a final concentration of 0.037% to all incubation mixtures. When extracts containing Tween 20 or DOC were analyzed, the final concentrations of these detergents also were adjusted to 0.037%. This concentration of these detergents has previously been shown not to affect virus attachment to HeLa cells. The mixtures were incubated for 1 h at 37°C on a shaking water bath and then centrifuged at 140 x g for 5 min at room temperature. The supernatants were transferred to round-bottom plastic tubes, and 2.5 x 10^6 previously EMEM-washed HeLa cells were added in a volume of 250 µl. This gave a final concentration of 5 x 10^5 cells/ml and a multiplicity of infection of about 340 particles/cell.

(ii) **Incubation 2.** The cell-containing mixtures were incubated for 1 h at 37°C as above, and samples were withdrawn at different intervals, diluted 10 times in EMEM, and centrifuged at 140 x g for 3 min. The radioactivity in the supernatants and the pellets was measured and used for calculating the degree of virus attachment. Three-point attachment curves were always constructed for the samples analyzed.

(iii) **Calculations.** The percentages of radioactivity remaining in the supernatants after 60 min of attachment were used to calculate the relative inhibition of virus attachment. This procedure was believed to counteract any possible variations in the status of the cells. Calculations were performed according to the formula:

\[
\text{relative inhibition} = \frac{\% \text{ cpm in supernatant (sample)}}{\% \text{ cpm in supernatant (control)}} \times 100
\]

\[
- \% \text{ cpm in supernatant (maximum inhibition)}
\]

\[
- \% \text{ cpm in supernatant (control)}
\]
where percentage of counts per minute in supernatant (sample) is the radioactivity remaining in the supernatant after 60 min of virus-cell interaction in the presence of presumptive receptor material; percentage of counts per minute in supernatant (control) is the radioactivity remaining in the supernatant after 60 min of virus-cell interaction in the absence of interfering material; and percentage of counts per minute in supernatant (maximum inhibition) is the radioactivity remaining in the supernatant at maximum inhibition of attachment in the presence of, for example, detergent-extracted plasma membrane proteins. The highest measured inhibition was 95% after 60 min of virus-cell interaction. The relative inhibition revealed by different quantities of Triton X-100 (0.5%) extracted proteins was determined, and a dose-response curve was constructed (see Fig. 3 in Results). We assumed that dose-response curves of similar shape would be obtained when using other extraction procedures. This would enable us to compare the efficiency of virus attachment inhibition of other protein extracts.

The degree of inhibition of all extracts was normalized to a 50% relative inhibition of attachment in the Triton X-100 system, using 0.5% detergent at extraction.

**Rate zonal sedimentation analysis of virus-attached plasma membrane proteins.** Highly purified Ad2 virions were mixed with solubilized plasma membrane proteins radioactively labeled in vitro. The amount of virus and protein used corresponded to multiplicities of infection between 6,000 and 8,000 particles per cell equivalent based on calculations of extraction efficiencies and original cell quantities used for plasma membrane preparations. The samples were incubated in the presence of 0.037% Triton X-100, 0.5% Triton X-100, 0.2 M NaCl, 1% BSA, or 3% BSA for 1 h at 37°C with shaking. Except for the incubation with 0.5% Triton X-100, all incubation mixtures were supplemented with Triton X-100 to give a final concentration of 0.037%. The mixtures were subsequently layered onto linear sucrose gradients consisting of 2 × 1.6 ml of 20 to 40% (wt/vol) sucrose in 50 mM Tris-hydrochloride buffer, pH 7.5, and containing the same amount of detergent, salt, or BSA as the pertinent incubation mixture. The gradients were underlaid with 0.2 ml of 60% (wt/vol) sucrose in the same buffer system and centrifuged at 100,000 × g for 40 min at 6°C. After fractionation into 25 fractions by puncturing from the bottom, radioactivity was monitored by counting 5-μl portions. Fractions of interest were pooled, dialyzed against double-distilled water, freeze-dried, and analyzed electrophoretically on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels.

**Chemical cross-linking of Ad2 virions.** Glutaraldehyde was used to stabilize Ad2 by cross-linking in the presence of 0.1 M sodium phosphate buffer, pH 7.4, in a procedure described by Avrameas and Ter-nyck (2) with some modifications. The concentrations of glutaraldehyde and virions were determined so as to maintain the virus particles in a true soluble state as well as provide the highest coupling efficiency of virions to AH-Sepharose 4B. In the procedure chosen, glutaraldehyde (25% solution) was added to a 5 optical density units solution of Ad2 in sodium phosphate buffer to make the concentrations of glutaraldehyde and sodium phosphate 5% and 0.1 M, respectively.

The total 3-ml volume of the mixture was incubated for 24 h at 8°C, whereafter it was freed of glutaraldehyde by filtering through a PD-10 column with Sephadex G-25M in 0.1 M sodium phosphate buffer, pH 7.4. Fractions were pooled after measuring the absorption at 280 nm. The stabilized Ad2 virions were further immobilized and used as an affinity adsorbent (see below).

**Fractionation of material with Ad2 virion affinity from the HeLa cell plasma membrane.** (i) **Wheat germ lectin-Sepharose 6MB chromatography of solubilized membrane proteins.** A column with 5 ml of wheat germ lectin-Sepharose 6MB was equilibrated with WGA buffer (0.2 M NaCl and 0.037% Triton X-100 in 0.05 M sodium phosphate buffer, pH 7.0). [3H]formaldehyde-labeled, Triton X-100 (0.5%)-solubilized membrane proteins were applied to the column after dialysis against WGA buffer for 18 to 20 h at 4°C. Fractions of 0.5 ml were collected at a flow rate of 3.4 ml/h. After the column was washed with WGA buffer, the retained proteins were eluted with N-acetyl-D-glucosamine (100 mg/ml) in WGA buffer. The elution profile was followed by direct counting of 10-μl portions from each fraction. The peak fractions of interest were pooled and dialyzed against WGA buffer for 20 h at 4°C.

(ii) **Affinity chromatography on an immobilized adenovirion matrix.** Highly purified Ad2 virions were stabilized by cross-linking as described above and incubated with carefully washed AH-Sepharose 4B (up to 2.5 optical density units of Ad2 per ml of gel) for 24 h at 4°C in a rolling state. Ethanolamine was added (1 M, final concentration) to block any residual aldehyde groups, and the mixture was incubated for another 12 to 15 h at 4°C. After the second incubation, the gel was washed extensively with WGA buffer and was then ready to use. By using radioactively labeled Ad2 virions, it was established that the coupling efficiency to AH-Sepharose 4B was 90 to 95%. Moreover, when coupled virions were treated with buffer solutions at low pH (0.2 M glycine-hydrochloride, pH 2.2), at high pH (0.2 M glycine-NaOH, pH 12.0), at high ionic strength (5 M MgCl2 in 50 mM Tris-hydrochloride, pH 7.4), and containing detergent (1% Triton X-100 in 50 mM Tris-hydrochloride, pH 7.4), no loss of virus was detected. Therefore, it was assumed that the viruses were covalently linked to the matrix.

Imobilized virions on an Ad2-AH-Sepharose 4B column were able to adsorb antifiber antibodies, indicating the presence of immunologically intact and accessible fibers of the virions. This immunological procedure was used to monitor the stability of the columns used throughout these investigations.

Unretained and retained fractions from the lectin chromatography step (see above) were analyzed on the Ad2-AH-Sepharose 4B (1.2 optical density units of Ad2 per ml of gel), equilibrated with WGA buffer. The pertinent fractions were applied to the column, and 3.2-ml fractions were collected at a flow rate of 3.4 ml/h. After washing of the column with WGA buffer, proteins of low virus affinity were eluted with WGA buffer containing 0.5 M NaCl. Proteins with high affinity for Ad2 virions were subsequently eluted with 0.1 M NaCl-0.037% Triton X-100 in 0.1 M glycine-
hydrochloride buffer, pH 2.6. Fractions obtained from elution at low pH were immediately neutralized with 1 M sodium phosphate buffer, pH 7.2. The elution profiles were monitored by direct radioactivity measurements of 50-μl portions withdrawn from each fraction. Fractions constituting the peaks of interest were pooled, dialyzed against double-distilled water, and freeze-dried.

**SDS-PAGE.** The SDS-polyacrylamide gel electrophoresis (PAGE) analyses were performed on gel slabs (1.5 by 70 by 70 mm) with 13% acrylamide and 0.38% bisacrylamide essentially as described by Maizel (30). Fluorography was done according to Bonner and Laskey (4).

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were done in a cocktail of toluene-methanol (1:1) with 0.4% Omnifluor. Assessment of trichloroacetic acid precipitable radioactivity was done on filter paper disks (Munktell, Grycksbo, Sweden, OOH; 21-mm diameter). Filters with added and dried samples were submerged into 10% ice-cold trichloroacetic acid for at least 30 min and then washed twice in 5% cold trichloroacetic acid and twice in 70% cold ethanol. The dried filters were counted in toluene with 0.4% Omnifluor. The 131I radioactivity was measured in the tritium channel with a 60% efficiency. A Nuclear-Chicago Mark II liquid scintillation counter was used to determine radioactivity.

Chemicals and radioisotopes. EMEM, L-glutamine, gentamicin, and fetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Scotland. [3H]-formaldehyde (0.1 Ci/mmole), [3H]thymidine (56 Ci/mmole), Bolton-Hunter reagent (125I; 2000 Ci/mmole), and Omnifluor were purchased from New England Nuclear Chemicals, GmbH, Dreieich, West Germany. N-acetyl-p-glucosamine, Triton X-100 (scintillation grade), DOC, and polyethylene glycol 6000 were from BDH Chemicals Ltd., Poole, England; Tween 20 and sodium cyanoborohydride were from Sigma Chemical Co., St. Louis, Mo. Wheat germ lectin-Sepharose 6MB, AH-Sepharose 4B, dxtran T-500, and PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. Kodak XG-14 X-ray films were used in the autoradiography and fluorography analyses and were developed in Kodak D-19, both products from Eastman Kodak Co., Rochester, N.Y. Glutaraldehyde (25% for electron microscopy) was from E. Merck AG, Darmstadt, West Germany.

**RESULTS**

Comparison of procedures for plasma membrane isolation. Two methods were studied for plasma membrane isolation as described in Materials and Methods. The recoveries of protein and distributions of marker enzymes are shown in Table 1. Based on the greater recovery of protein, the higher enrichment of 5'-nucleotidase activity, the facts that the two-phase separation procedure is faster, less laborious, and more suitable in a preparative scale, this technique was chosen for further plasma membrane preparations (6). The purity of the plasma membranes prepared by this method was controlled in a phase-contrast microscope and electron microscope, and no contamination of intact cells, nuclei, mitochondria, or granular elements was observed (data not shown).

Solubilization of plasma membrane proteins. The efficiencies of membrane protein release by various functionally distinct solubilization techniques were compared (Table 2). Some extractions were performed in such a way that one solubilization technique was followed by another in order to release material from the nonextractable remainder of the previous procedure.

### Table 1. Comparison of methods for plasma membrane isolation

| Method and sample | Protein recovery (%) | 5'-Nucleotidase | Na⁺,K⁺-ATPase | NADPH cytochrome c reductase |
|-------------------|----------------------|-----------------|-----------------|-------------------------------|
|                   |                      | Sp act (μmol of PO₄⁻³/h and mg) | Relative sp act | Sp act (μmol of PO₄⁻³/h and mg) | Relative sp act | Sp act (μmol/h and mg) | Relative sp act | Recovery (%) |
| Boemann et al. (5) | Homogenate           | 17 ± 1.1 (3)    | ND              | ND                            | ND              |
|                   | Supernatant 50.4 ± 36.2 (3) | 12 ± 0.9 (3)    | 0.9 ± 0.4 (3)   | 0.29 ± (5)                    | ND              |
|                   | Fraction II 1.5 ± 0.17 (3) | 4.7 ± 1.8 (3)   | 3.6 ± 1.9 (3)   | 0.49 ± 0.09 (4)               | 0.56 ± 0.18 (4) |
| Brunette and Till (7) | Homogenate 100        | 1.4 ± 0.8 (6)   | 0.4 ± 0.2 (3)   | 1.17 ± 0.37 (4)               | 1.62 ± 0.28 (4) |
|                   | Supernatant 62.5 ± 6.0 (13) | 1.4 ± 0.4 (3)   | 0.8 ± 0.2 (3)   | 10.5 ± 8.4 (5)                | 10.5 ± 8.4 (5)  |
| Interphase'       | 3.9 ± 0.9 (23)       | 9.3 ± 4.9 (6)   | 7.3 ± 2.8 (6)   | 2.10 ± 1.1 (5)                | 10.5 ± 8.4 (5)  |

* Values are expressed as mean ± standard deviation; number of experiments is given in parentheses. NADPH, Reduced nicotinamide adenine dinucleotide phosphate; ND, not determined.

* Described in Materials and Methods.

* In the two-phase separation system, the plasma membranes are enriched in the interphase.
procedure. It is evident that the detergent methods released the highest quantities of membrane protein. The high-salt and the organic solvent procedures were one-third to one-half as efficient.

When we compared the total polypeptide contents of the high-salt and the detergent extracts in SDS-polyacrylamide slab gels, we found that the solubilization by detergents enriched a number of proteins compared with the starting material (Fig. 1). Qualitatively, all extracts displayed similar polypeptide patterns. The electropherogram also supported the general concept about the versatility of proteins constituting the protein moiety of the HeLa cell plasma membrane (8).

Identification of receptor-active material. Solubilized plasma membrane proteins obtained from the high-salt, organic solvent, and detergent extraction procedures were analyzed for the presence of material interfering with the process of adenovirus attachment to HeLa cells. A prerequisite for this analysis was the development of an assay for receptor-active material and its subsequent normalization (see Materials and Methods). Initially, the kinetics of adenovirus attachment to HeLa cells was studied (Fig. 2). After approximately 40 min of virus-cell interaction, maximum attachment was attained. The increased values for the standard deviation of later measuring points could have resulted from physical stress on the cells after this time of incubation. The tendency of the function to decrease at later time points could have been due to the fact that virions at this stage of incubation had entered the interior of the cells (26) and thus avoided detection, since the samples were assayed for radioactivity directly and not hydrolyzed before analysis. The basis for the normalized relative inhibition calculations was the dose-response curve (Fig. 3). Compared with total membrane content, a significant amount of receptor-active material (i.e., material interfering with virus attachment) was present in the 0.5% Triton X-100 extracts. Resuspension of the nonextractable remainder provided a sample with low ability to inhibit virus attachment, and although extreme quantities were used, only a maximum relative inhibition of around 25% was achieved. Table 2 shows the efficiencies of various other plasma membrane protein extracts in the inhibition assay. The Triton X-100 procedure using 0.5% detergent gave a 50% relative inhibition of virus attachment, using the lowest amount of extracted protein. Extraction with this concentration of detergent also made it possible to perform complete dose-response analyses without removing the detergent (see Materials and Methods). To simplify the comparison of Triton X-100 extracts with other extracts, all estimates were normalized to the Triton X-100 (0.5%) system (Table 2). Although the DOC procedure gave good production of receptor-active material, it was not considered for further studies because it could strongly interact with proteins irreversibly. The 3 M KCl extraction method produced an extract with poor inhibitory effect. We tried to optimize the Triton X-100 extraction procedure by first removing membrane components of low interfering ability with 3 M KCl and thereafter apply the Triton X-100 system to the nonsolubilized remainder. This sequence of extractions yielded an extract with only half the efficiency of the standard Triton X-100 (0.5%) procedure (Table 2).

Identification of polypeptides attaching to adenovirions. Since the Triton X-100 (0.5%) method produced an extract with the highest inhibitory effect on virus attachment to HeLa

| Table 2. Receptor activity (RI) of solubilized plasma membrane proteins recovered by different procedures* |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Extraction procedure | Protein recovery (%) | Amt of protein needed for 50% RI (μg) | Amt of protein needed for 50% RI normalized to the 0.5% Triton X-100 extraction system (μg) |
|----------------------|----------------------|-----------------------------------|-------------------------------------------------|
| 3 M KCl              | 17.6 ± 3.0 (4)       | 150 (1)                           | 3.1                                             |
| 3 M KCl-10% pyridine | 4.5 ± 0.8 (2)        | ND                                | ND                                              |
| Triton X-100         | 46.0 (1)             | 84 (1)                            | 1.8                                             |
| 10% n-Butanol        | 12.0 (1)             | ND                                | ND                                              |
| 10% n-Pentanol       | 17.7 (1)             | ND                                | ND                                              |
| 10% Pyridine         | 20.8 ± 4.7 (5)       | 74 ± 17.6 (2)                     | 1.5 ± 0.4                                       |
| 10% Pyridine-10% n-pentanol | 0 (1) | ND                                | ND                                              |
| 1.0% DOC             | 69.7 (1)             | 59 (1)                            | 1.2                                             |
| 0.1 % Triton X-100   | 25.2 (1)             | NE (1)                            | ND                                              |
| 0.5 % Triton X-100   | 37.6 ± 4.9 (14)      | 48 ± 13.2 (25)                    | 1.0 ± 0.2                                       |
| 1.0% Triton X-100    | 43.9 ± 6.9 (3)       | 52 ± 7.1 (2)                      | 1.1 ± 0.2                                       |
| 0.5% Triton X-100    | 71 ± 26.9 (2)        | ND                                | ND                                              |
| - PD-10*             | -                    | NE (10)                           | ND                                              |
| 0.5% Tween 20        | 18.8 ± 6.3 (3)       | NE (5)                            | 1.5 ± 0.6                                       |
| 1.0% Tween 20        | 30.6 ± 6.8 (4)       | NE (5)                            | ND                                              |
| Total membranes      | 86 ± 19.6 (11)       | 1.8 ± 0.4                          | ND                                              |

* The relative inhibition of virus attachment was calculated as described in Materials and Methods. Where applicable, values are followed by standard deviation, with the number or experiments given in parentheses. ND, Not determined; NE, no effect (highest amount of protein analyzed was 80 μg).

A second extraction of the nonextractable remainder of the first was done as described in Materials and Methods.

Before analysis in the attachment assay, the protein extracts were deprived of free detergent by passage through a PD-10 column followed by extensive dialysis.
cells, this technique was chosen for further investigations. Solubilized and in vitro $^{125}$I-labeled plasma membrane proteins were incubated with highly purified Ad2 virions as described in Materials and Methods. The incubated mixtures were subsequently layered onto sucrose gradients and sedimented as described. Reproducibility about 0.3% of the added radioactivity cosedimented with the virus particles in the presence of 0.037% Triton X-100 (Fig. 4). If the detergent concentration of the incubation mixture and gradient was increased to 0.5% Triton X-100, about 0.2% of the total radioactivity still remained associated with the virus. An additional increase of the detergent concentration up to 1% did not further reduce the amount of material attached to virions. In some instances, a low degree of virion and protein aggregation was discerned (0.06% of the total radioactivity) in the presence of both 0.037 and 0.5% Triton X-100. However, the majority of sedimenting labeled protein was always located in the region of the virus control. When 1 or 3% BSA was added to the incubation mixtures and gradients, we observed no membrane proteins specifically cosedimenting with virions. When this large amount of protein was added (50 to 150 times more BSA per virus particle than membrane protein per virus particle), virus and membrane protein seemed to aggregate nonspecifically. Approximately 2.4% of the extracted membrane proteins were aggregated throughout the entire gradient when 3% BSA was used, compared with about 0.7% with 1% BSA.

Fractions from the sucrose gradient analyses of protein cosedimenting with virions were pooled as indicated in Fig. 4 and subjected to SDS-PAGE analysis as described in Materials and Methods. About 13 polypeptide bands were found associated with virions in the presence of 0.037% Triton X-100 (Fig. 5A). One of the predominant polypeptides had a molecular weight of approximately 40,000. The addition of 0.2 M NaCl had no effect on the qualitative pattern of polypeptides associated with the virus particles (data not shown), although the amount of proteins associated with virions was reduced to about 0.1%. If the detergent concentration was
increased to 0.5%, the 40,000-dalton polypeptide was the predominant species associated with virions, together with four faintly observable polypeptides (Fig. 5B). This 40,000-dalton polypeptide was probably identical to the protein species adsorbed with the highest affinity to the Ad2–AH-Sepharose 4B matrix (see below).

Lectin chromatography of solubilized plasma membrane proteins. Triton X-100 (0.5%)-solubilized and \( ^{3} \text{H} \)formaldehyde-labeled membrane proteins were fractionated on wheat germ lectin-Sepharose 6MB as described in Materials and Methods. Radioactivity measurements reproducibly established that 5% of the total amount of protein material applied was retained by the column (pool B, Fig. 6).

Immobilized Ad2 as an adsorbent. Because adenovirus particles are fragile (25, 35), they must be stabilized before being immobilized and used as an adsorbent. Proteins of the virus particles were therefore cross-linked with glutaraldehyde and immobilized as described in Materials and Methods. These procedures preserved the ability of virions to interact with antifiber immunoglobulin G. Virions immobilized with no spacer reacted to a limited extent with antifiber antibodies, whereas the introduction of a six-carbon spacer produced a functional adsorbent (not shown).

Unretained and retained fractions of the Triton X-100-solubilized and radioisotope-labeled plasma membrane proteins from the wheat germ lectin-Sepharose 6MB chromatography step were analyzed in a column of immobilized Ad2 virions. Figure 7 shows the elution pattern when unretained material from the lectin chromatography (pool A, Fig. 6) was applied to the column. Of the radioactive material, 95% had no affinity for Ad2 and could be washed out with WGA buffer. Of the remaining 5% of radioactivity, 3% was eluted with WGA buffer containing 0.5 M NaCl, whereas the radioactivity still bound was eluted only after a drastic decrease in pH (see Materials and Methods).

When membrane proteins possessing N-acetyl-D-glucosaminyl residues (pool B, Fig. 6) were fractionated on the Ad2 column, a totally different pattern was obtained. Only 41% of the total radioactivity could be washed out unadsorbed with WGA buffer (Fig. 7B). Of the remaining
proteins analyzed for self-aggregation particles. Symbols: (---) Radioactivity of Triton X-100 (0.5%)-solubilized plasma membrane proteins, \(^{125}\text{I}\) labeled in vitro and mixed with Ad2 virions; (---) radioactivity of solubilized, iodine-labeled proteins analyzed for self-aggregation in a separate centrifuge tube; (...) UV absorbance (260 nm) of Ad2 particles sedimented in a separate centrifuge tube as a sedimentation control for the system. A typical experiment with 0.037% Triton X-100 in incubation mixtures and gradients is shown, details of which are described in Materials and Methods. Sedimentation was from right to left. The horizontal bars indicate fractions pooled for further analysis (see Fig. 5).

59%, 38% was eluted at high ionic strength (0.5 M NaCl) and 21% was eluted at low pH (2.6). This means that about one-fifth of the wheat germ lectin-adsorbed proteins had high affinity for Ad2, which further corresponds to about 1% of the totally solubilized membrane protein content. This figure should be compared with the 0.3% of solubilized membrane proteins adsorbed to Ad2 virions in the sucrose gradient system.

Polypeptide analysis of the virus-retaining material. From the virus affinity chromatograms, the different peak fractions were pooled as indicated in the Fig. 7 and further analyzed on SDS-polyacrylamide slab gels (Fig. 8). Because of the low amount of radioactivity applied, patterns for sample wells with several polypeptides were difficult to observe. The pooled fractions from pool B in the lectin chromatography step (Fig. 6) and with high affinity for Ad2 virions (pool B3 of Fig. 7B) revealed one heavily and one poorly labeled polypeptide band with apparent molecular weights of 42,000 and 40,000, respectively. The analogous fractions derived from pool A (Fig. 6 and 7A) showed two equally labeled polypeptide bands with the same apparent molecular weights as those of pool B3 (Fig. 7B).

DISCUSSION

The plasma membrane fraction of HeLa cells has been isolated preparatively, and by means of various extraction procedures some information has been obtained on the nature of the Ad2 attachment proteins and their association with the plasma membrane.

Assuming that the components that are important for the initial attachment step of viruses to cells are loosely associated surface entities of the plasma membrane, high-salt conditions should be effective in releasing this material. The 3 M KCl extraction procedure used here is well established for these purposes (33), but a high-salt extract of plasma membranes had little effect in the relative inhibition assay (Table 2). Presumably due to denaturing conditions of the high-salt procedure or the removal of a cooperating attachment factor, a consecutive Triton X-100 detergent extract also had little effect in the receptor assay. The results indicate that the attachment proteins are localized as integral entities of the plasma membrane. This is further supported by the fact that removal of the free detergent from a 0.5% Triton X-100 extract decreased the inhibitory activity (Table 2).

That the assayed components were integrated structures of the membranes was further indicated by the observation that an extract of the nonionic detergent Tween 20 did not display any measurable receptor activity (Table 2). One explanation for the lack of receptor release with this detergent could be the higher hydrophilic-lipophilic balance value for Tween 20 (16.7) than for Triton X-100 (13.5). It is generally believed that hydrophilic-lipophilic balance values above 14.5 are associated with lower ability to solubilize integral membrane proteins (15, 17). Of course, the different configurations of the detergents, i.e., size and structure of the polar and apolar moieties of the detergent molecules, could also contribute to the distinct extraction properties. Conformational changes of the attachment proteins cannot be ruled out, but to circumvent this possibility the receptor assay was developed and an extraction procedure was eventually selected that yielded receptor-active material in a soluble state.

Hennache and Boulanger (18) described the isolation of proteins interfering with the attach-
ment of Ad2 to KB cells. Fibers and total penton structures of the virions, immobilized by various techniques, were used as adsorbents. By using total Ad2 virions, we have allowed for the subsequent identification of all possible membrane components of stronger as well as weaker affinity for all surface structures of the intact Ad2 virion.

We noticed a quantitative difference in the amount of extracted proteins associated with virions under various conditions. In the sucrose gradient system, the attachment efficiency ranged between 0.2 and 0.3% when Triton X-100 was present at 0.5 and 0.037%, respectively. Membrane proteins were in these instances added in amounts ranging from $2 \times 10^{-10}$ to $3 \times 10^{-10} \mu g$ per virion. Ten- to 100-times-lower ratios of protein over virions were used in the immobilized system, and under these conditions as much as 3% of the solubilized protein content was adsorbed. Of this material, one-third (i.e., 1%) was retained with high affinity. The calculations were based on pertinent fractions, taking into account all possible technical considerations. Meager et al. (31) and Hughes and Mautner (21) have suggested the involvement of glycoproteins during virus-cell interaction, and our preliminary data also suggest that glycosylated components are of interest. Therefore, fractionation on WGA-Sepharose was done to drastically concentrate the glycoprotein content of the total membrane extracts; in this way, samples relatively rich in receptor activity were obtained for the subsequent fractionations on the immobilized virion particle matrix. It should be emphasized that this prefractionation was not done before the assays in the sucrose gradient system, and this fact could explain the lower yield of virus-attaching proteins in the gradient analyses compared with the Ad2 matrix system. Supporting data for the idea of nonspecific protein competition may also come from experiments where large quantities of added proteins irrelevant for the virus-cell attachment step, e.g., BSA, strongly affected the attachment of membrane...
proteins to virions. These findings are contradictory to the results of Hughes and Mautner (21), who in their not fully comparable system of purified fibers and membrane components were not able to affect the fiber-receptor recognition.

The analyses in sucrose gradients and on immobilized virus particles apparently show at least two types of affinity of membrane proteins to virus particles. We focused here on the high-affinity fraction, but it is possible that proteins with lower affinity for viruses still may be involved in the attachment step. The high-affinity fraction obtained by the two procedures is dominated by a radiolabeled polypeptide with an apparent molecular weight of 42,000. One of the Ad2 attachment polypeptides on KB cells enriched by Hennache and Boulanger (18) displayed the same molecular weight (18).

Based on the elution profiles from the affinity chromatographies on WGA and Ad2 matrices (Fig. 6 and 7), we suggest that the high-affinity proteins contain N-acetyl-d-glucosaminy1 residues. This means that the indicated molecular weights may be uncertain, because SDS-PAGE is not the optimal system to determine the molecular weight of glycoproteins (14). The autoradiogram patterns for pools A3 and B3 (Fig. 8) were very similar, and the visible bands probably corresponded to the same polypeptide. However, with respect to the different affinities for WGA, the carbohydrate moieties of the polypeptides in A3 and B3 must have been distinct, since the elution patterns were not caused by overloading artefacts during chromatography. The different behaviors may be due to a partial degradation of the carbohydrate moiety or incomplete glycosylation during the biosynthesis of the polypeptides recovered in pool A3 (28).

Since it has been reported that the receptors for Ad2 are sparsely represented on human cell plasma membranes (21), we have worked with membrane proteins radiolabeled in vitro to identify the components of the attachment site. It is not possible to obtain the same specific radiolabeling of all polypeptides in the plasma mem-

**Fig. 6.** Wheat germ lectin-Sepharose 6MB chromatography of Triton X-100-solubilized and in vitro [3H]formaldehyde-labeled plasma membrane proteins of HeLa cells. A typical experiment is shown in which 1.4 x 10^5 cpm of radioactivity was applied to the column and eluted as described in Materials and Methods. The arrow indicates the start of elution with WGA buffer containing N-acetyl-d-glucosamine (100 mg/ml). The horizontal bars (A and B) represent fractions pooled for further studies (see Fig. 7).

**Fig. 7.** Affinity chromatography on an immobilized Ad2 matrix. (A) Pool A, from the wheat germ lectin-Sepharose 6MB chromatography separation (Fig. 6) was applied to a column of cross-linked Ad2 virions immobilized to AH-Sepharose 4B and fractionated as described in Materials and Methods. The filled arrow indicates the start of elution with WGA buffer containing 0.5 M NaCl, and the open arrow indicates the start of elution with 0.1 M NaCl-0.037% Triton X-100 in 0.1 M glycine-hydrochloride buffer, pH 2.6. The horizontal bars represent the peak fractions pooled and are denoted A1, A2, and A3 according to elution order. (B) Pool B from the previous wheat germ lectin-Sepharose 6MB chromatography separation (Fig. 6) was fractionated as described above.
brane. One must therefore consider that some polypeptides may escape being labeled. Thus, our preliminary results with unlabeled HeLa cell plasma membrane proteins reveal similar patterns when chromatographed on WGA and Ad2 matrices. However, in these instances the A3 and B3 pools, upon SDS-PAGE analysis, display distinct polypeptide patterns after staining of the electropherograms. Moreover, it has been demonstrated that very low quantities of unlabeled proteins of pool B3 interfere with the Ad2 attachment to cells (R. Persson, U. Svensson, and E. Everitt, manuscript in preparation).

ACKNOWLEDGMENTS

The excellent technical assistance provided by Blanka Boberg is gratefully acknowledged.

This investigation was supported by grants from the Swedish Cancer Society, Carl Tryggers Foundation; Helge Axson Johnson's Foundation; and Magnus Bergvall's Foundation, all in Stockholm, Sweden.

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