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# Complex Formation of Class I Transplantation Antigens and a Viral Glycoprotein*

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**Olle Kämpe, Donald Bellgrau‡, Ulf Hammerling, Peter Lind, Svante Pääbo, Liv Severinsson, and Per A. Peterson**

*From the Department of Cell Research, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden*

**Indirect immunoprecipitations of labeled glycoproteins from the adenovirus-transformed rat cell line $A_2T_2C_4$ and from adenovirus-infected HeLa cells revealed that the class I major histocompatibility antigens co-precipitated with the viral E19 protein. The degree of co-precipitation was highly dependent on the antiserum used. The identity of the co-precipitated components was verified by peptide mapping and radiochemical amino acid sequencing. Cell-free translation of mRNA for the E19 protein and the class I antigen heavy chains demonstrated that the E19 protein-class I antigen interaction is an inherent property of the participating components. In intact cells the virus protein and the transplantation antigens form large complexes, held together by weak, noncovalent interactions.**

T-cells can only recognize foreign antigens in conjunction with major histocompatibility complex structures (1). Cytotoxic T-cells preferentially recognize foreign antigens in the context of class I molecules, HLA-A, B, and C antigens in man and H-2K, D, and L antigens in the mouse (2), while helper T-cells primarily react towards antigens presented together with class II molecules, HLA-DR antigens in man and Ia antigens in the mouse (3). The extensive genetic polymorphism of the class I and II antigens, as well as their structural similarity with immunoglobulins (4–7), raise the possibility that class I and II antigens may function as primitive cell surface antibodies (8). If this idea has any merit one may expect to find physical complexes of major histocompatibility complex antigens and foreign molecules on the cell surface. Several controversial reports have argued for the existence of such complexes (see Refs. 9–16).

We have shown that an early adenovirus glycoprotein, the E19 protein, forms complexes with class I antigens (14). In this article we extend this observation and demonstrate by identification at the structural level that the E19 protein forms complexes with class I antigens both in transformed and acutely infected cells. Moreover, we demonstrate that isolation of the complexes depends on a judicious choice of the antiserum used.

## EXPERIMENTAL PROCEDURES AND RESULTS[1]

*Complex Formation between Class I Antigen Chains and the E19 Protein Following Cell-Free Translation*—We examined whether a spontaneous association of class I antigen and E19 protein chains occurs. To this end, mRNAs coding for class I antigen heavy and light chains and the E19 protein were isolated from Raji cells and infected HeLa cells, respectively, and translated *in vitro* in the presence of dog pancreas microsomes.

All three mRNA preparations were efficiently translated, and the immunoreactive chains were in each case completely confined to the microsomal vesicles. The mRNA preparations for the class I antigen heavy chains and the E19 protein were co-translated in the presence of varying amounts of microsomes. In the presence of high concentrations of microsomes, no co-precipitation could be demonstrated. However, on lowering the concentration of microsomes, antibodies against the heavy chain co-precipitated the E19 protein (Fig. 1). Antibodies against the E19 protein, on the other hand, did not co-precipitate significant amounts of the heavy chain (not shown, *cf.* Table I).

These data suggest that class I antigen heavy chains and the E19 protein may associate independently of other plasma membrane constituents.

*Efficiency of Various Antisera to Co-Precipitate Class I Antigens and the E19 Protein*—We examined the co-precipitation of class I antigens and the E19 protein using various antisera. To this end, [$^{35}$S]methionine-labeled glycoproteins of $A_2T_2C_4$ cells and adenovirus-infected HeLa cells were immunoprecipitated. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the amount of label associated with the constituent chains of the complex was measured. Table I summarizes the results. Although the degree of co-precipitation varied with the same antiserum in different experiments, the data of Table I are representative.

Table I shows that the two reagents against class I antigen heavy chains reacted similarly with material from the $A_2T_2C_4$ cells. Although the heavy chains were precipitated in different amounts the co-precipitations of the E19 protein and the $\beta_2$-

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‡ Present address, The Basel Institute of Immunology, Basel, Switzerland.

[1] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1–6, and Table I) are presented in miniprint at the end of the paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-153, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press. The abbreviations used are: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SDS sodium dodecyl sulfate; HPLC high pressure liquid chromatography; BPB, bromphenol blue; TCA, Trichloroacetic acid.

microglobulin, expressed in relative amounts, were low and similar. The antiserum against $\beta_2$-microglobulin co-precipitated a significant amount of the heavy chain but only a fraction of the E19 protein. The antiserum against the virus protein precipitated approximately 10-fold more of this protein than did any of the co-precipitating antisera (Table I). Taken together, these results demonstrate that the degree of co-precipitation of the associated polypeptide chains vary with the antiserum.

Table I also summarizes the data obtained with infected HeLa cells. Also in this case, the relative amounts of the three precipitated chains vary. A monoclonal antibody raised against cell-surface expressed $\beta_2$-microglobulin was most efficient in co-precipitating heavy chains and the E19 protein. In fact, this antibody precipitated more of the virus protein than did the antiserum raised against the E19 protein.

Common to all precipitations was the finding that labeled $\beta_2$-microglobulin appeared to be present in smaller amounts in the E19 protein-producing cells than in the control cells (*cf.* Fig. 3). This may signify that the synthesis of $\beta_2$-microglobulin or that the size of the endogenous pool of $\beta_2$-microglobulin changes when the E19 protein is expressed. Alternatively, the affinity of $\beta_2$-microglobulin for the heavy chain may decrease on complex formation (see "Discussion").

## DISCUSSION

The present study confirms and extends previous observations that class I antigens and an early adenovirus-coded glycoprotein, the E19 protein, form complexes (14, 16). Thus, in this article we demonstrate that monoclonal antibodies directed against class I antigen chains co-precipitate the E19 protein both from adenovirus-transformed and -infected cells, thereby eliminating the possibility that the previously demonstrated co-precipitations were the result of extraneous antibodies present in the rabbit antisera used.

Since the isolation of class I antigen-virus protein complexes in general has been hard to document, it seemed of importance to ascertain at the structural level that the immunological co-precipitations involved class I antigens and the E19 protein. Thus, the 40,000-Da chains precipitated by antisera against class I antigens and co-precipitated by an antiserum against the E19 protein were shown to be identical by peptide mapping. Likewise, radiochemical amino acid sequence analyses of the 19,000-Da component co-precipitated by class I antigen antisera revealed that it is identical with the E19 protein of the adenovirus E3 region (17, 18).

The immunological co-precipitations suggested that the protein complexes only consisted of class I antigens and the E19 protein. As an initial attempt, we wished to examine whether the complex formation was dependent on other plasma membrane or cytoplasmic components. Moreover, we also wished to determine whether both or only one of the class I antigen chains was needed for the complex formation. To this end, the mRNAs coding for the E19 protein and the class I antigen chains were translated *in vitro* in the presence of varying concentrations of microsomal vesicles. Complex formation occurred at low but not at high concentrations of the vesicles. In the latter case, the polypeptide chains most probably were sequestered in separate vesicles. Although the immunoprecipitations were carried out subsequent to the solubilization of the vesicles, which should have brought the various polypeptide chains into contact, this was obviously not sufficient to generate complexes. From these observations it seems reasonable to conclude that complex formation may be dependent on a high local concentration of the associating proteins. In addition, the spatial orientation of the interacting components may also be of importance, but complex formation does not seem to be greatly dependent on extraneous components, *e.g.* cytoskeletal elements.

During the course of this study, it was noted that different antisera with the same nominal specificity varied in their efficiency to precipitate the class I antigen, E19 protein complexes. This notion was substantiated by quantitatively examining the ability of several antisera to precipitate the protein complexes both from adenovirus-transformed and -infected cells. The most likely interpretation of the co-precipitation data is that some of the antibodies, particularly those against the heavy chains and the E19 protein, respectively, may bind to structures directly involved in the complex formation (19). In the extreme, such antibodies should either be nonreactive or dissociate the protein complexes.

Despite the fact that antibodies against $\beta_2$-microglobulin co-precipitated heavy chains and the E19 protein quite efficiently, the protein complexes isolated with these antibodies, as well as with antibodies against the heavy chains, contained less labeled $\beta_2$-microglobulin than expected from precipitates of control cells. The interpretation of this observation is obscure. Thus, the intracellular pool of $\beta_2$-microglobulin (20–22) may change during an adenovirus infection or the rate of synthesis of $\beta_2$-microglobulin may be reduced in E19 protein-expressing cells as compared to control cells. A more intriguing interpretation is, of course, the possibility that all heavy chains of the protein complex do not associate with $\beta_2$-microglobulin. Provided that the complexes consist of many copies of the heavy chain (see below), only a fraction of those would have to be bound to $\beta_2$-microglobulin to ascertain the co-precipitations observed. Thus, a conformational change of the heavy chain, induced by the interaction with the virus protein, may diminish the affinity for $\beta_2$-microglobulin.

It is obvious that the apparent molecular weight and chain composition of the protein complexes are difficult to determine since antibody binding as well as dilution caused by solubilizing the cells may dissociate the complexes. For this reason, we covalently cross-linked the proteins prior to the immunoprecipitations to minimize dissociations. Complexes isolated by this procedure were so large that we could not estimate their apparent molecular weights. Despite the fact that several molecules must make up the complexes, de-cross-linking did not reveal the presence of components other than the class I antigen subunits and the E19 protein.

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SUPPLEMENTARY INFORMATION TO:

**COMPLEX-FORMATION OF CLASS I TRANSPLANTATION ANTIGENS AND A VIRAL GLYCOPROTEIN**

By

**Olle Kämpe, Donald Bellgrau, Ulf Hammerling, Peter Lind, Svante Pääbo, Liv Severinsson and Per A. Peterson**

## EXPERIMENTAL PROCEDURES

**Animals, cells and virus.** The adenovirus-transformed rat fibroblast line $A_2T_2C_4$, derived from Hooded Lister rat embryos (23), as well as Hooded Lister rats were kindly supplied by Dr P.H. Gallimore. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (GIBCO, Glasgow, U.K.), 100 U/ml of penicillin and 100 µg/ml of streptomycin. HeLa cells, obtained from the American Type Culture Collection, were grown in roller bottles in Eagle's medium supplemented with 2% fetal calf serum and antibiotics as above. Adenovirus type 2, kindly donated by Dr G. Wadell, was purified according to Green and Wold (24) although the pH was kept at 7.4 throughout the purification procedure. Adenovirus infections were carried out with approximately 10,000 virus particles per HeLa cell in a serum-free medium. After one hour the serum-containing medium was added to the infected cells.

**Antibodies.** The rabbit antiserum raised against highly purified $H\text{-}2^d$ antigens was the same as described earlier (25). Antiserum against highly purified urinary rat $\beta_2$-microglobulin, kindly supplied by Drs L. Björk and L. Lögdberg, was raised in a rabbit. The rabbit antisera raised against human HLA-A, B and C antigens and against human urinary $\beta_2$-microglobulin, respectively, were the same as earlier described (26). A monoclonal antibody against human $\beta_2$-microglobulin (R.9.11.4) was obtained from Dr J. Rosenlund (Pharmacia Fine Chemicals, Sweden). A rat monoclonal antibody against rat class I antigen heavy chains was produced using conventional fusion and cloning protocols (see ref. 27). A monoclonal antibody of similar specificity was the kind gift of Dr D. Smilek.

**Chemicals and enzymes.** The sources of the chemicals were: acrylamide, N.N'-methylenebisacrylamide, and Triton X-100 from Eastman Kodak Co. (Rochester, N.Y.), phenyl-methylsulfonyl fluoride (PMSF), dithiothreitol (DTT) Tris (Trizma grade) from Sigma Co. (St. Louis, Mo.), and dithiobis (succinimidyl) proprionate (DTSP) from Pierce Chemical Co. $^{35}$S-Methionine (900-1200 Ci/mmol), $^3$H-(75 Ci/mmol) and $^{14}$C-(0.5 Ci/mmol)-phenylalanine were the products of the Radiochemical Centre (Amersham, U.K.). Oligo-dT-cellulose was from Collaborative Research, (Waltham, Mass.). Bovine serum albumin, three times crystallized, and TPCK-treated trypsin were products of Worthington Enzymes. $^3$H-Lysine (80 Ci/mmol) was from New England Nuclear (Boston, Mass.).

**Radioactive labeling of cells** $A_2T_2C_4$ cells in log-phase were incubated for 6 hours in methionine-, lysine-, or phenylalanine-free DMEM supplemented with 4 mM glutamine, 5% dialysed fetal calf serum, 1% normal DMEM and the appropriate radioactive amino acid. Adenovirus-infected HeLa cells were radioactively labeled in the same medium between 3 and 7 hours after the initiation of the infection.

**Immunoprecipitation.** Radioactively labeled cells were solubilized in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1% Triton X-100, 1 mM PMSF and 1% Trasylol. After 30 min at 0° nuclei and cell debris were removed by centrifugation at 100,000 xg for 30 minutes at +4°C. The supernatants were enriched for glycoproteins on Lens Culinaris hemagglutinin - Sepharose 4B columns (28). Glycoproteins were eluted by the addition of 10% α-methylmannoside in the lysis buffer. Immunoprecipitation on the eluate from the lectin-column was performed as previously described (20). When using the monoclonal antibody R9.11.4, a rabbit anti-rat immunoglobulin serum was used as a second antibody to ascertain binding to the bacteria.

The resulting immunoprecipitates were collected using the Staphylococci. The pelleted bacteria were subsequently washed 4 to 5 times with ice-cold 0.02 M Tris-Hcl buffer, pH 7.4, containing 0.15 mNaCl and 0.2% Triton X-100. The immunoprecipitates were desorbed from the bacteria by heating at 95°C for 3 minutes in the SDS-polyacrylamide gel electrophoresis sample buffer which consisted of 0.10 M Tris-HCl buffer, pH 8.8, containing 3% SDS, 30% sucrose, 10 mM DTT and trace amounts of BFB. When the bacteria had been removed by centrifugation, iodoacetamide was added to a final concentration of 50 mM. When immunoprecipitations were performed with in vitro translation products, protein A-Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as the insoluble matrix rather than the bacteria.

**Tryptic peptide mapping.** Spleen cells from Hooded Lister rats were labeled with $^3$H-phenylalanine, and $A_2T_2C_4$ cells were labeled with $^{14}$C-phenylalanine. Immunoprecipitations of labeled glycoproteins from the cells were performed with various antisera and the polypeptide chains of interest were fractionated on cylindrical SDS-polyacrylamide gels, which after completed electrophoresis were segmented into 2-mm slices. The proteins were eluted and localized by their content of radioactivity. Proteins to be compared by peptide mapping were pooled and TCA-precipitated together with 150 µg of crystalline bovine serum albumin (Sigma Co., St. Louis, Mo.). The precipitates were washed twice with a diethyl ether-ethanol (1:1 v/v) and then dissolved in 100 µl of 0.2 M ammonium bicarbonate, pH 8.1, containing 4 M urea. To the clear solution 5 µg of TPCK-trypsin were added in 100 µl of 0.1 M ammonium bicarbonate. After 90 min another 5 µg portion of TPCK trypsin was added. The digestion was terminated by heating at 95°C for 3 min after a total incubation period of 4 hours. Peptides were separated on a $C_{18}$ (Bondapak) reverse phase column using a 0 to 80% ethanol gradient in 0.1 M ammonium acetate. 0.8 ml fractions were collected at 1 min intervals. Radioactivity in the eluate was measured in a Packard Tri-Carb scintillation counter with a double-isotope program. Quench-corrections were made by the external standard channel ratio method.

**Radiochemical amino acid sequence determination.** Radiochemical amino acid sequence determinations were carried out on immunoprecipitated and electrophoretically purified material derived from labeled cells. Cylindrical SDS-polyacrylamide gels were segmented and minced by a Gilson automatic gel slicer after completion of the electrophoretic separation. When the segments containing the relevant protein had been identified by radioactivity measurements on aliquots from each segment, the protein was eluted by soaking the appropriate segments overnight in 20 mM of Tris-HCl buffer, pH 8.0, containing 0.3% SDS. The radioactive protein was reduced and alkylated in a 0.2 M Tris-HCl-buffer, pH 8.0, containing 0.3% SDS. One mg of crystallized bovine serum albumin was added to the sample prior to the precipitation of the protein with ice-cold TCA at a final concentration of 20%. The precipitate, collected by centrifugation, was washed twice in a diethylether-ethanol (1:1 v/v) to remove SDS and TCA from the protein. Precipitates were dissolved in 0.5 ml of trifluoroacetic acid and subjected to automatic amino acid sequencing in a Beckman 890 C sequencer. After drying, a wash cycle, omitting the addition of the cleavage acid, was carried out. The fast protein Quadrol Program with 0.5 M Quadrol was used in all analyses (19). The anilinthiazolinones were dried under a stream of $N_2$ and converted to phenylthiohydantoins in 0.2 ml of 1 M HCl containing 0.1% ethanethiol at 80°C for 10 minutes. The phenylthiohydantoin derivatives were extracted with two 0.7 ml-portions of ethylacetate, dried under $N_2$ and subjected to radioactivity measurement in a Packard Tri-Carb Liquid scintillation counter.

**Isolation of mRNA.** Microsomes were isolated (21) from exponentially growing Raji cells and from infected HeLa cells harvested at 9 hours after the initiation of an adenovirus-infection. RNA was extracted from the microsomal fraction with phenol-chloroform-isoamyl alcohol (25:24:1 v/v) and the mRNA was enriched by oligo-dT-cellulose chromatography. The mRNA was further purified on a 10 to 30% linear aqueous sucrose gradient by centrifugation for 11 hours at 39,000 rpm in a Beckman SW 40 rotor at 15°C. Fractions containing mRNA corresponding to human class I antigen heavy chains, $\beta_2$-microglobulin and the E19 protein were identified by cell-free translation (see below) and immunoprecipitation. The mRNA fractions were stored at -70°C until used.

**Cell-free translation.** Translation of mRNA in vitro was performed in a rabbit reticulocyte lysate system according to Pelham and Jackson (29). Dog pancreas microsomes, kindly supplied by Dr B. Dobberstein, were added to a concentration of 1 $A_{280}$ unit/ml. The lysates were incubated at 30°C for 90 min. To isolate microsomes after translation, KCl was added to 100 mM and EDTA to 5 mM. The microsomes were sedimented through a 10% sucrose cushion by centrifugation in a Beckman Airfuge for 5 minutes. The resulting pellets were dissolved in a 0.02 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 2 mM EDTA and 1% Triton X-100.

**Crosslinking.** Glycoprotein fractions from radioactively labeled cells in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.147 M NaCl, 0.003 M KCl and 0.2% Triton X-100 were incubated with $2\times10^{-4}$ M DTSP for 30 minutes at room temperature. The crosslinking reaction was terminated by the addition of lysine to a final concentration of 2 mM. Separation of the proteins from the crosslinking reagent was performed on a small Sephadex G-200 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Immunoprecipitations of crosslinked proteins were performed as described above. However, the following changes were introduced to avoid reducing the crosslinker: elution of immune complexes from the bacteria was accomplished without heating and the sample buffer did not contain DTT. De-crosslinking was accomplished by reduction with DTT and boiling.

**Other methods.** SDS-polyacrylamide gel electrophoresis was carried out as described by Blobel and Dobberstein (30) on 10 to 15% gradient slab gels. Cylindrical gels, using a 12% acrylamide concentration, were run according to Laemmli (31). Slab gels were fixed in 10% TCA for 15 minutes and then treated with Enhancer (New England Nuclear, Boston, Mass.) for 60 minutes. Subsequently, the gels were soaked in water containing 1% glycerol for 20 to 30 minutes. Fluorography was accomplished as described by Bonner and Laskey (32) on dried gels. Kodak XAR films were used throughout.

## RESULTS

Table I

Reactivity of various antibodies with $^{35}$S-methionine-labeled glycoproteins from $A_2T_2C_4$ cells, normal rat spleen cells, adenovirus-infected and mock-infected HeLa cells[1]

| Antiserum[2] | Amount precipitated from $A_2T_2C_4$ cells | | | Ratio[3] | Amount precipitated from spleen cells | | Ratio[3] |
|---|---|---|---|---|---|---|---|
| | E19 | H | $\beta_2$M | E19:H:$\beta_2$M | H | $\beta_2$M | H:$\beta_2$M |
| | cpm | | | | cpm | | |
| R anti-HR | 630 | 8.000 | 1.300 | 6:100:16 | 5.400 | 3.200 | 100:60 |
| MC anti-HR | 280 | 1.500 | 160 | 8:100:4 | 1.500 | 660 | 100:43 |
| R anti-$\beta_2$MR | 730 | 3.000 | 5.900 | 20:100:213 | 3.300 | 4.600 | 100:148 |
| R anti-E19 | 6.300 | 1.800 | 220 | 403:100:7 | 240 | 100 | - |
| R NS | 180 | 280 | 110 | - | 290 | 140 | - |

| Antiserum[2] | Amount precipitated from infected HeLa cells | | | Ratio | Amount precipitated from normal HeLa cells | | Ratio |
|---|---|---|---|---|---|---|---|
| | E19 | H | $\beta_2$M | E19:H:$\beta_2$M | H | $\beta_2$M | H:$\beta_2$M |
| | cpm | | | | | cpm | |
| R anti-HH | 5.000 | 11.000 | 1.200 | 45:100:10 | 7.600 | 1.800 | 100:24 |
| R anti-$\beta_2$MH | 6.000 | 6.800 | 1.500 | 91:100:21 | 4.200 | 2.100 | 100:52 |
| MC anti-$\beta_2$MH | 14.400 | 9.800 | 1.700 | 152:100:17 | 9.800 | 2.500 | 100:25 |
| R anti-E19 | 10.400 | 2.600 | 220 | 477:100:3 | 300 | 120 | - |
| R NS | 240 | 470 | 150 | | 370 | 100 | - |

[1] Aliquots of the glycoprotein fractions of the labeled cells were separately immunoprecipitated with the various antisera. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis. The amounts of radioactivity in the class I antigen heavy (H) and light ($\beta_2$M) chains and in the E19 protein were estimated by subjecting 1.0 mm slices of the gels to a liquid scintillation counter.

[2] The antisera used were: Rabbit antiserum against murine class I antigen heavy chains (R anti-HR), monoclonal antibodies against rat class I antigen heavy chains (MC anti-HR), rabbit antiserum against rat $\beta_2$M (R anti-$\beta_2$MR), rabbit antiserum against the E19 protein (R antis-E19), rabbit antiserum against human class I antigen heavy chains (R anti-HH), rabbit antiserum against human $\beta_2$M (MC anti-$\beta_2$MH). R NS denotes normal rabbit serum.

[3] The ratios were calculated by setting the radioactivity of class I antigen heavy chains to 100 in all precipitates. The radioactivities in the E19 protein and $\beta_2$M peaks respectively, were expressed in percentages of the radioactivity of class I antigen heavy chains peaks. All values have been corrected for back-ground.

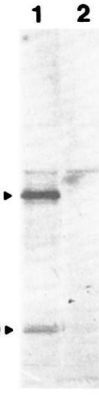


Figure 1
Co-precipitation of class I antigen heavy chains and the E19 protein in vitro
Sucrose gradient-fractionated mRNAs specifying the E19 protein, and the HLA-A, B and C antigen heavy chains respectively, were mixed and subjected to cell-free translation in vitro in the presence of dog pancreas microsomes (1 $A_{280}$ unit of microsomes per ml) and $^{35}$S-methionine. The translation products present in the microsomes were immunoprecipitated with rabbit antiserum against human class I antigens (lane 1) and with a control serum (lane 2). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and radioactive bands were detected by fluorography.

Transplantation antigens and a virus encoded protein form a ternary complex in adenovirus-transformed $A_2T_2C_4$ cells and in acutely adenovirus-infected HeLa cells

The adenovirus-transformed rat fibroblast line $A_2T_2C_4$ has integrated about 80% of the adenovirus genome, and several of the early proteins are expressed (17). Previously we showed that indirect immunoprecipitation of $^{35}$S-methionine-labeled glycoproteins of $A_2T_2C_4$-cells using a rabbit antiserum against class I antigens brought down a 19,000 dalton polypeptide, in addition to the two expected class I antigen chains (14). To rule out that the rabbit antiserum contained extraneous antibodies directed against the adenovirus protein the experiment was repeated using two different rat monoclonal antibodies against the class I antigen heavy chain. Fig. 2 shows that both reagents co-precipitated the 19,000 dalton chain from $^{35}$S-methionine-labeled $A_2T_2C_4$ cells. However, the 19,000 dalton chain was not observed when the immunoprecipitations were performed with the same monoclonal antibodies on material derived from primary rat fibroblasts (Fig. 2, lane 3). This suggests that the 19,000 dalton chain is of viral origin and confirms that a complex between transplantation antigens and a viral protein exists in $A_2T_2C_4$ cells.

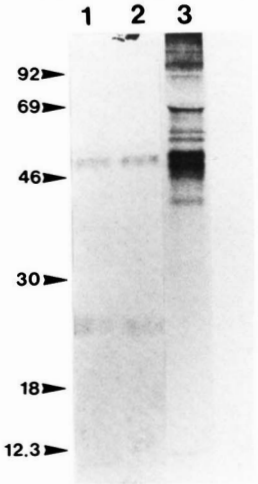


Figure 2
Co-precipitation of class I antigens and the E19 protein in the adenovirus transformed celline $A_2T_2C_4$.
Fluorography following SDS-polyacrylamide gel electrophoresis of $^{35}$S-methionine labeled molecules from $A_2T_2C_4$ cells (lanes 1 and 2) precipitated with two different monoclonal antibodies recognizing rat class I heavy chains. In lane 3 a mixture of these antibodies was used to precipitate $^{35}$S-methionine labeled molecules from rat fibroblasts. The arrows denote the migration of marker proteins. The molecular weights are given in kilodaltons. The E19 protein migrates abberantly due to its glycosylation and appears as a 25,000 dalton protein.

HeLa cells are permissive for human adenovirus-2, i.e. the virus that was used to establish the $A_2T_2C_4$ cell line. Productively infected and mock-infected HeLa cells were labeled with $^{35}$S-methionine and solubilized glycoproteins were subjected to immunoprecipitation. A rabbit anti-human class I antigen serum precipitated a 19,000 dalton chain together with the heavy and light transplantation antigen chains from the infected cells (Fig. 3, lane 1) but not from the mock-infected cells (Fig. 3 lane 4). Both rabbit antiserum and monoclonal antibodies against human $\beta_2$-microglobulin precipitated the same set of molecules as the antiserum against the class I antigens (Fig. 3, lanes 2, 3, 5 and 6). Thus, it may be concluded that antisera directed against both the heavy and the light chains of the class I molecules co-precipitate a 19,000 dalton virus protein.

The 19,000 dalton virus-coded polypeptide chain could also be precipitated by an antisem raised against the purified virus glycoprotein both from $A_2T_2C_4$ cells (14) and from adenovirus-infected but not mock-infected HeLa cells. In both cases class I antigens were co-precipitated (not shown).

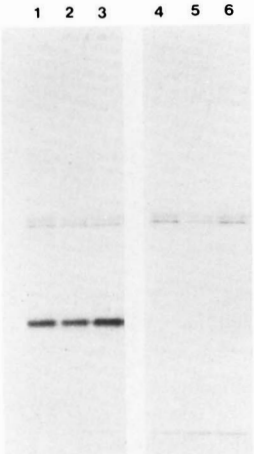


Figure 3
Co-precipitation of class I antigens and the E19 protein in adenovirus- but not mock-infected HeLa cells.
Fluorography of an SDS-polyacrylamide slab-gel showing immunoprecipitates obtained from $^{35}$S-methionine-labeled, adenovirus-infected (lanes 1-3) or mock-infected (lanes 4-6) HeLa cells. The rabbit sera used were directed against HLA, B and C antigens (lanes 1 and 4) and against human $\beta_2$-microglobulin (lanes 2 and 5). A rat monoclonal antibody (R.9.11.4) raised against human $\beta_2$-microglobulin (lanes 3 and 6) was also used.

Identification of the constituent chains of the class I antigen-virus protein complex derived from $A_2T_2C_4$ cells

The polypeptides precipitated from $A_2T_2C$ cells by a rabbit anti class I antigen serum were compared to class I antigens of syngeneic spleen cells. The immunoprecipitates, containing $^3$H- and $^{14}$C-labeled phenylalanine, respectively, were separately resolved by SDS polyacrylamide gel electrophoresis. The 44 000 dalton chains were recovered from the gels, mixed, digested with trypsin and subjected to reverse phase HPLC. Fig. 4 shows that the two 44 000 dalton chains were identical as regards their phenylalanine-containing, tryptic peptides.

In a separate experiment the tryptic peptides of the 44,000 dalton chain, isolated by co-precipitation with the E19 protein from $A_2T_2C_4$ cells, were shown to be identical to the tryptic peptides of the class I antigen heavy chains of $A_2T_2C_4$ cells (not shown). Likewise, the 44,000 dalton chain co-precipitated with the E19 protein from adenovirus-infected HeLa cells displayed a peptide pattern indistinguishable from that of class I antigen heavy chains of the mock-infected cells.

The complete nucleotide sequence of the E19 gene (31) in conjunction with limited radiochemical protein sequence information on the signal sequence of the E19 protein (32) have established the entire amino acid sequence of the protein. We examined whether the E19 protein of $A_2T_2C_4$ cells, which co-precipitated with class I antigens, is identical to the E19 gene product. $A_2T_2C_4$ cells were separately labeled with $^3$H-lysine and $^3$H-phenylalanine and the E19 protein was isolated by immunoprecipitation of the class I antigens. Following SDS-polyacrylamide gel electrophoresis the E19 protein was recovered from the gel and subjected to automatic amino acid sequence determination. The identification of phenylalanines in positions 6 and 15 and of lysines in positions 2, 3 and 7 (Fig. 5) is in agreement with the amino acid sequence predicted from the nucleotide sequence.

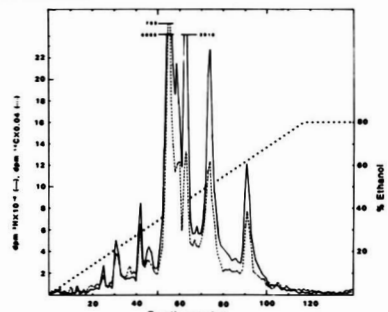


Figure 4
Comparison of the 44,000 dalton chain co-precipitated with the E19 protein and class I molecules of spleen cells
High-pressure liquid chromatography on a Bondapac C18 column of tryptic digests of 44,000 dalton material from $^3$H-phenylalanine-labeled Hooded Lister spleen cells and $^{14}$C-phenylalanine-labeled $A_2T_2C_4$ cells, respectively. The glycoprotein fractions of the labeled cells were separately isolated by affinity chromatography on Lens Culinaris hemagglutinin column. Molecules were immunoprecipitated with an antiserum against class I antigens. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis on cylindrical gels and polypeptide chains with apparent molecular weights of 44,000 were recovered, mixed and subjected to tryptic digestion. Details are given under Experimental Procedures.

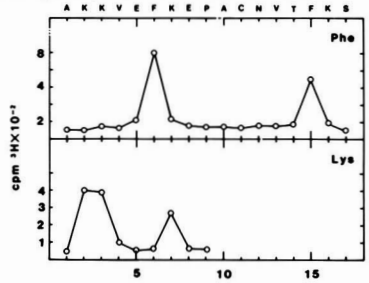


Figure 5
The 19,000 dalton polypeptide is encoded in the adenovirus-2 EcoRI-D region
The 19,000 dalton polypeptide was isolated by immunoprecipitation of material from $A_2T_2C_4$ cells, labeled with $^3$H-lysine and $^3$H-phenylalanine, respectively, using an antiserum specific for class I antigens. Further purification was achieved by SDS-polyacrylamide gel electrophoresis. Radiochemical sequence determination was performed as described under Experimental Procedures. The $NH_2$-terminal amino acid sequence predicted from the nucleotide sequence of the adenovirus-2 EcoRI-D fragment is given on top in the one-letter code. This sequence has been shown to code for an adenovirus glycoprotein with a molecular weight of 19,000 (31).

In a control experiment the E19 protein of $A_2T_2C_4$ cells was isolated with the antiserum raised against the E19 protein and subjected to amino acid sequence determination. The result was identical to that documented in Fig. 5.

These data firmly identify the 44 000 and 19 000 dalton polypeptides forming complexes in $A_2T_2C_4$ cells as heavy chains of class I antigens and the E19 protein of adenovirus-2, respectively.

Large class I antigen-E19 protein complexes are revealed by covalent crosslinking

Since antibodies seem to affect the stoichiometry of the class I antigen chains and the E19 protein in the complexes (see Table I) we used the covalent crosslinker DTSP to reduce dissociation during the immunoprecipitations. To this end labeled glycoproteins from $A_2T_2C_4$ cells and adenovirus-infected HeLa cells, respectively, were crosslinked, immunoprecipitated and separated by SDS polyacrylamide gel electrophoresis. Crosslinked proteins of $A_2T_2C_4$ cells (Fig. 6B) and infected HeLa cells (Fig. 6E) precipitated by antisera against class I antigen heavy chains remained at the top of the gels while immunoprecipitates of non-crosslinked proteins gave the expected electrophoretic patterns (Fig. 6A and D). The high molecular weight components of Fig. 6B and E were separately eluated, de-crosslinked and re-examined by SDS polyacrylamide gel electrophoresis (Fig. 6C and F). Only class I antigen chains and the E19 protein were resolved after de-crosslinking with a slight change in their relative propotions

These observations strongly suggest that adenovirus-infected and transformed cells contain fragile class I antigen-E19 protein complexes of large sizes. Moreover, the data support the notion that these complexes exclusively consist of class I antigen chains and the E19 protein.

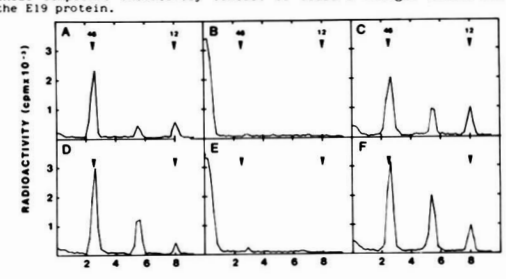


Figure 6
Covalent crosslinking of class I antigen-E19 protein
The glycoprotein fractions of $^{35}$S-methionine labeled $A_2T_2C_4$ cells (A-C) and adenovirus-infected HeLa cells (D-F) were separately treated with the cleavable crosslinker DTSP. Immunoprecipitations were carried out with rabbit antisera specific for murine (A-C) and human (D-F) class I antigen heavy chains, respectively, and the precipitated molecules were separated by SDS-polyacrylamide gel electrophoresis. A and D display immunoprecipitates obtained prior to the crosslinking. B and E show the immunoprecipitates recovered after crosslinking of the glycoproteins and C and F demonstrate the electrophoresis pattern after de-crosslinking of the immunoprecipitates. The arrows denote the migration positions of the marker proteins where molecular weights are given in kilodaltons.


# Complex formation of class I transplantation antigens and a viral glycoprotein.

O Kämpe, D Bellgrau, U Hammerling, P Lind, S Pääbo, L Severinsson and P A Peterson

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