Purification of HLA-A2 Antigen, Fluorescent Labeling of Its Intracellular Region, and Demonstration of an Interaction between Fluorescently Labeled HLA-A2 Antigen and Lymphoblastoid Cell Cytoskeleton Proteins in Vitro†

Jordan S. Pober,* Braydon C. Guild, Jack L. Strominger, and William R. Veatch

ABSTRACT: Detergent-solubilized HLA-A and -B antigens (p44,12) have been purified from lymphoblastoid cell membranes by means of allospecific monoclonal antibody immunoaffinity chromatography. Papain-solubilized HLA-A and -B antigens (p34,12) which lack the transmembrane and intracellular portions of the HLA heavy chain have previously been purified to homogeneity by this technique [Parham, P. (1979) *J. Biol. Chem. 254*, 8709–8712]. In contrast, preparations of HLA-A and -B antigens isolated from detergent extracts of total lymphoblastoid cell membranes by the method of Parham invariably contain a substantial amount of actin (5–50%, depending on the preparation). The actin is tightly bound to the detergent-solubilized HLA-A and -B antigens and could not be removed from the solubilized complex without denaturation or proteolysis of the proteins. Detergent-solubilized HLA-A and -B antigens free of actin can be prepared, however, by selective extraction of cytoskeletal proteins from the membrane prior to detergent extraction of the HLA-A and -B antigens. Detergent-solubilized HLA-A and -B antigens contain an easily reduced cysteine residue(s) whereas papain-solubilized antigens do not; i.e., the cysteine residue(s) is located within the transmembrane or intracellular portion of the HLA heavy chain [Springer, T. A., & Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2481–2485]. Purified detergent-solubilized HLA-A2 antigen may be fluorescently alkylated in its intracellular region with *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (IAEDANS), and the AEDANS moiety is confirmed to be attached to the intracellular portion of the molecule by using limited proteolysis. Fluorescence excitation, emission, and polarization measurements indicate that the AEDANS reporter group is sensitive to the HLA-A2 protein conformation. Furthermore, the AEDANS reporter group detects an interaction when fluorescently labeled HLA-A2 is recombined with lymphoblastoid membrane derived cytoskeletal proteins. This interaction does not occur when AEDANS-mercaptoethanol (a model compound) is substituted for the HLA-A2 antigen or when other proteins (interacting and noninteracting antibodies) are substituted for the cytoskeletal proteins. Finally, the cytoskeletal proteins have been partially fractionated, and the interaction with HLA antigens appears specific for cytoskeletal proteins that partition with a polymerized form of actin. These results offer the opportunity to study membrane protein–cytoskeletal interactions in vitro.

HLA-A and -B antigens are transmembrane proteins found in the plasma membrane of all human cells except erythrocytes. These molecules have a two-chain structure; the light chain (p12 or β_2-microglobulin) is invariant in all HLA-A and -B antigens and forms a noncovalent complex (p44,12) with the alloantigenically variable heavy chain (p44) (Springer & Strominger, 1976). The heavy chain of native HLA-A and -B antigens has three principal regions: a large glycosylated extracellular amino-terminal region (p34) that is complexed to the light chain, a penultimate hydrophobic region of approximately 25 residues that spans the membrane, and a hydrophilic carboxy-terminal intracellular region of approximately 30 residues. Papain sequentially releases the hydrophilic carboxy terminus and then the hydrophobic penultimate region from native HLA-A and -B antigens in a stepwise fashion, yielding p39,12 and p34,12 complexes, respectively (Springer & Strominger, 1976). Trypsin releases only the hydrophilic carboxy-terminal region, yielding a molecule (p39,12) that is similar to the papain intermediate product (Engelhard et al., 1978). The amino acid sequence of the intracellular region has been determined for the HLA-B7 antigen and tentatively assigned for the HLA-A2 antigen (Robb et al, 1978). The HLA-B7 molecule contains two easily reduced and alkylated (by iodoacetamide) cysteine residues, one of which is lost upon papain release of the carboxy-terminal hydrophilic intracellular region and the other of which is lost upon subsequent papain release of the penultimate transmembrane hydrophobic region. These cysteine residues have been identified within the sequence of the HLA-B7 antigen. The HLA-A2 antigen contains an easily reduced and alkylated residue(s) that is removed completely upon the first step of papain cleavage or upon trypsin cleavage; i.e., it is located within the carboxy-terminal intracellular region of the HLA-A2 heavy chain (Robb, 1977). The precise location (and identity) of the alkylated residue(s) of HLA-A2 was not identified in the tentative sequence of the carboxy-terminal region of this molecule. However, the carboxy-terminal CNBr fragment of HLA-A2 (or HLA-B7) labeled biosynthetically with [^{35}S]cysteine is radioactive (unpublished experiments), indicating that a cysteine residue is present and strongly suggesting that the easily reduced and alkylated residue is indeed a cysteine. Furthermore, under mild (nondenaturing, pH 8.1) conditions of reduction and alkylation, the disulfide-bonded cysteine residues of the extracellular portions of HLA-A2 (or HLA-B7) remain as cystine and are not modified, so that *only the intracellular cysteine residue(s) becomes alkylated* (Springer & Strominger, 1976). In the work reported here, allospecific monoclonal antibodies have been used to purify milligram quantities of detergent-solu-

† From The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138 (J.S.P., B.C.G., and J.L.S.), and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 (W.R.V). *Received January 23, 1981.* Supported by research grants from the National Institutes of Health (AI-10736 and AM-13230 to J.L.S. and AI-15311 to W.R.V.) and by a National Institutes of Health fellowship to J.S.P. W.R.V. is the recipient of Research Career Development Award AI-00320. A report of some of this work has been presented at the 1980 Annual Meeting of the Biophysical Society, New Orleans, LA.

* Address correspondence to this author at the Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115.

0006-2960/81/0420-5625$01.25/0

bilized HLA-A2 (and -B7) antigens from JY lymphoblastoid cells (doubly homozygous HLA-A2, -B7) by immunoaffinity chromatography. Furthermore, *N*-[[(iodoacetyl)amino]-ethyl]-5-naphthylamine-1-sulfonic acid (IAEDANS),[1] a fluorescent iodoacetamide derivative introduced by Hudson & Weber (1973), has been used to fluorescently alkylate HLA-A2 antigen selectively in the carboxy-terminal intracellular portion of the HLA heavy chain. We have chosen to modify HLA-A2 because this modification probably results in single-site labeling, whereas mildly reduced HLA-B7 is alkylated at two sites.

Several lines of evidence suggest that HLA-A and -B antigens interact with cytoskeletal proteins in vivo. Double immunofluorescence microscopy has been used to show that the arrangement of membrane proteins such as H2-K and -D antigens (murine homologues of HLA-A and -B) on the cell surface is coincident with the arrangement of submembranous cytoskeletal proteins in a variety of cell types including lymphocytes (Bourguignon et al., 1978). Furthermore, H2-K and -D antigens appear to be specifically associated with actin in shed fragments of membrane (Koch & Smith, 1978). More recently, the arrangement of HLA-A and -B antigens has also been shown by double immunofluorescence microscopy to be coincident with that of cytoskeletal proteins in human fibroblasts (Huet et al., 1980) and in lymphoblastoid cells (B. C. Guild and J. L. Strominger, unpublished experiments). The lateral mobility of HLA-A and -B antigens on the cell surface, as determined by fluorescence photobleach recovery, has been shown to be retarded at least 13-fold relative to that of lipid, consistent with cytoskeletal restraint of the mobility of HLA-A and -B antigens (Petty et al., 1980). The structural basis of these membrane protein–cytoskeletal interactions is unknown. In the erythrocyte, a distinct membrane binding protein has been identified for spectrin, the major erythrocyte cytoskeletal component, and this cytoskeletal–membrane interaction may be reconstructed in vitro [reviewed by Lux (1979)]. The spectrin system is unique to the erythrocyte, and cytoskeletal protein–membrane protein interactions have not been reconstituted in more complex cell types. We report here that the HLA-A2 antigen, fluorescently labeled specifically in its carboxy-terminal intracellular region, interacts with lymphoblastoid cytoskeletal proteins when recombined in vitro as revealed by a change in the fluorescence signal of the reporter group. This system offers an opportunity to study interactions of a membrane protein with cytoskeletal elements in vitro and may be used to explore the structural basis and regulation of this interaction.

Experimental Procedures

Materials. Detergents used in the purification of fluorescently labeled HLA antigens were Brij (a 2:1 mixture of Brij 99 and 97) from ICI United States, Nonidet P-40 (NP40) from Particle Data Laboratories, and deoxycholate (DOC) from Schwarz/Mann. Dithiothreitol (DTT), adenosine 5′-triphosphate (ATP), phenylmethanesulfonyl fluoride (PMSF), quinine sulfate, and *N*-[[(iodoacetyl)amino]methyl]-5-naphthylamine-1-sulfonic acid (IAEDANS) were from Sigma. Both iodo[^{3}H]acetic acid and Aquasol were from New England Nuclear. Sodium dodecyl sulfate ($NaDodSO_4$) and other reagents for polyacrylamide gel electrophoresis came from Bio-Rad. DEAE-Sepharose and Sepharose CL-4B were from Pharmacia, *Lens culinaris* agglutinin (lentil lectin) was from Vector, and both normal human serum and the allospecific monoclonal antibodies (PA2.1 and BB7.1) were a generous gift from Dr. Peter Parham. TPCK-trypsin was from Worthington. All other chemicals were reagent grade.

Preparation of Affinity Resins. Normal human immunoglobulin was prepared from serum and allospecific mouse monoclonal antibodies from ascites fluid by 50%-saturated ammonium sulfate precipitation and dialysis against 150 mM NaCl and 10 mM sodium phosphate, pH 7.4. The immunoglobulins as well as lentil lectin were coupled to Sepharose CL-4B using cyanogen bromide activation of the resin by the buffer method of Cuatrecasas (March et al., 1974) at a coupling density of 1–2 mg of protein/mL of packed Sepharose.

Purifications of Monoclonal Antibodies for Spectroscopy. PA2.1 and BB7.1 (the allospecific mouse monoclonal antibodies reactive against HLA-A2 and -B7, respectively) were purified from ascites fluids by 50%-saturated ammonium sulfate precipitation, by dialysis against 10 mM Tris-HCl, pH 8.0, and by chromatography on DEAE-Sepharose using 10 mM Tris-HCl, pH 7.5, loading buffer and a NaCl gradient in the same buffer to elute the antibodies.

Purification of HLA-A2 and -B7 Antigens. All solutions used in the purifications of HLA-A and -B antigens contained 0.1 mM PMSF, and all steps were performed at 4 °C. Frozen (at −70 °C) JY lymphoblastoid cells (50 g) (doubly homozygous HLA-A2, -B7) were thawed and hypotonically lysed, and membranes were harvested as described (Parham et al., 1977). The final membrane pellet was homogenized in 100 mL of 10 mM Tris-HCl, pH 8.0, layered in two tubes over 20 mL of 10% sucrose in 10 mM Tris-HCl, pH 8.0, and spun through the sucrose for 2 h at 100000*g*. The sucrose-washed pellet was homogenized in 100 mL of 10 mM Tris-HCl, pH 8.0, and the membranes were collected by centrifugation (20 min at 40000*g*) and resuspended (by vortexing) in 100 mL of 1 mM $(Na)_2EDTA$, pH 7.4. The membranes were again collected by centrifugation (20 min at 40000*g*) and rewashed with 1 mM $(Na)_2EDTA$, pH 7.4, as before. The second pellet was homogenized in 100 mL of ATP–DTT–low-salt extraction buffer (2 mM Tris-HCl, pH 8.0, 200 μM NaATP, 200 μM $MgCl_2$, 500 μM DTT, and 0.002% sodium azide) and dialyzed (M_r 12 000 cutoff) against 2 L of the same solution for 14–20 h. The membranes were harvested by centrifugation (20 min for 40000*g*), and the supernatant (the crude cytoskeletal extract) was set aside on ice. The membrane pellet was washed twice with a 1 mM solution of EDTA, pH 7.4, as before, and the final pellet was homogenized in 80 mL total volume of 12.5 mM Tris-HCl, pH 8.0. A 20-mL sample of 20% Brij was added, and the membranes were gently stirred for 30 min at 4 °C. The solution was spun for 2 h at 100000*g*, and the clarified supernatant was carefully removed from the detergent-insoluble residue in the pellet.

The supernatant was applied to a series of 12-mL columns recently purged with 50 mM diethylamine and 0.2% NP40 and then preequilibrated in 0.1 M Tris-HCl, pH 8.0, and 1.0% NP40. The columns were, in sequence, Sepharose CL-4B, normal human immunoglobulin–Sepharose CL-4B, BB7.1–Sepharose CL-4B (an allospecific mouse monoclonal antibody reactive toward HLA-B7 coupled to Sepharose), and PA2.1–Sepharose CL-4B (an allospecific mouse monoclonal antibody reactive toward HLA-A2 coupled to Sepharose). The Brij-

[1] Abbreviations used: IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid; AEDANS, the fluorescent carboxymethyl moiety of IAEDANS; AEDANS-ME and AEDANS-A2, the fluorescent products of alkylation of β-mercaptoethanol and HLA-A2 antigen by IAEDANS, respectively; Brij, a 2:1 mixture of Brij 99 and Brij 97 nonionic detergents; DOC, deoxycholate; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; NP40, Nonidet P-40 nonionic detergent; PAS, periodic acid–Schiff staining method; PMSF, phenylmethanesulfonyl fluoride; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

extracted proteins were washed through the series of columns by an additional 40 mL of 0.1 M Tris-HCl, pH 8.0, and 1% NP40, and the entire effluent was collected as the "flow through". The column series was then separated, and each monoclonal antibody column was separately washed with 10 column volumes of 20 mM Tris-HCl, pH 8.0, and 0.2% NP40; these effluents were pooled as the "wash". Each column was then separately eluted into one 6-mL and three 12-mL fractions using 50 mM diethylamine (pH 11.5) and 0.2% NP40. Each fraction was immediately neutralized by addition of 0.1 volume of 2 M Tris-HCl, pH 8.0. The purged columns were reequilibrated by passage of 0.1 M Tris-HCl, pH 8.0, and 1% NP40 and were stored in this buffer.

The HLA-A2 and -B7 antigen containing fractions were concentrated on a PM30 Amicon filter by pressure dialysis and dialyzed (using a M_r 12000 cutoff membrane) against 10 mM Tris-HCl, pH 8.0. The concentrated antigens (~1000 μg of protein/mL) were stored frozen at −20 °C prior to fluorescent labeling.

Fluorescent Labeling of HLA-A2 Antigens. A 1.0-mL aliquot of the purified HLA-A2 antigen was thawed, made 2.5 mM in DTT, and incubated for 1 h at 37 °C. The sample was cooled to room temperature and made 100 mM in Tris-HCl, pH 8.1. Sufficient solid IAEDANS to make the solution 10 mM was added and dissolved in the dark. The solution was incubated in the dark for 1 h at room temperature. β-Mercaptoethanol (10 μL) was then added to quench the reaction.

Excess fluorescent reagent was removed by *Lens culinaris* (lentil lectin) affinity chromatography (Springer et al., 1974). A 2.0-mL lentil lectin–Sepharose column was equilibrated with 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.05 mM $CaCl_2$, 0.05 mM $MnCl_2$, and either 0.1% DOC or 0.5% Brij. The fluorescent reaction mixture was applied, and five 5.0-mL fractions were collected. (This was sufficient to reduce the fluorescence in the effluent so that it was not detectable with a hand-held UV lamp.) The fluorescent HLA-A2 antigen was then eluted by addition of 4% methyl α-mannoside to the column buffer; 10 mL of eluate containing approximately 500 μg of labeled protein was collected and dialyzed against 150 mM NaCl and 10 mM Tris-HCl, pH 8.0, containing detergent (either 0.1% DOC or 0.5% Brij). On occasion, the labeled protein was concentrated prior to dialysis using a PM30 Amicon filter.

Proteolysis of AEDANS-Labeled HLA-A2. AEDANS-labeled HLA-A2 purified by lentil lectin affinity chromatography was divided into four aliquots of 0.1 mL, each containing 10 μg of protein, and 0.01 mL of 10 mM Tris-HCl, pH 8.0, containing 0, 0.05, 0.2, or 1.0 μg of TPCK-trypsin (Worthington) was added to each, respectively. The samples were incubated 30 min at 37 °C. The reaction was stopped by cooling the samples to 4 °C and adding 1 mM PMSF in absolute ethanol. The samples were then precipitated by addition of 12% trichloroacetic acid and prepared for $NaDodSO_4$–polyacrylamide gel analysis (see below).

Preparation of Cytoskeletal Proteins. A number of extrinsic membrane proteins, enriched in cytoskeletal proteins such as actin, were prepared from JY lymphoblastoid cell membranes. A crude cytoskeletal extract was obtained from the dialysis of washed membranes against ATP–DTT–low-salt extraction buffer (2 mM Tris-HCl, pH 8.0, 200 μM NaATP, 200 μM $MgCl_2$, 500 μM DTT, 0.002% sodium azide, and 0.1 mM PMSF). The extracted proteins were collected in the supernatant after a 20-min 40000*g* centrifugation. The extract was further clarified by ultracentrifugation (2 h at 100000*g*) and concentrated 10× by pressure dialysis on an Amicon PM30 membrane. The concentrated extract sometimes became cloudy and was clarified by overnight dialysis against 1 L of the ATP–DTT–low-salt extraction buffer and subsequent ultracentrifugation (3 h at 100000*g*). This supernatant (designated "total cytoskeletal concentrate") was carefully removed from the pellet. A portion was set aside and stored at 4 °C; the remainder was further fractionated by formation of actin polymers. This was performed by making the total cytoskeletal concentrate 1 mM $CaCl_2$ and either 80 mM KCl (low KCl concentration) or 800 mM KCl (high KCl concentration) and incubating at room temperature for 1 h. These solutions were respun (3 h at 100000*g*), and the supernatants (designated "actin-poor supernatants") were carefully separated from the translucent pellets (designated "actin-rich pellets"). The actin-rich pellets were redissolved in ATP–DTT–low-salt extraction buffer, and both fractions (the actin-poor supernatants and the actin-rich pellets) were dialyzed 2 times for 24 h against 1 L of ATP–DTT–low-salt extraction buffer. The total cytoskeletal concentrate, the actin-poor supernatants (high and low KCl concentration) and the actin-rich pellets (high and low KCl concentration) were stored in ATP–DTT–low-salt extraction buffer at 4 °C until ready for use, at which time they were dialyzed against 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$.

Preparation of Samples for Spectroscopy. Fluorescently labeled HLA-A2 (AEDANS-A2) in 0.25% Brij or 0.1% DOC was prepared as described above and diluted with its final dialysis buffer to the desired concentration (usually 5–10 μg of protein/mL). The IAEDANS adduct of β-mercaptoethanol (AEDANS-ME) was prepared by addition of 10 μL of β-mercaptoethanol to 0.5 mg of IAEDANS in the same dialysis buffer used to equilibrate the AEDANS-A2. Samples were made 8 M in urea by addition of solid crystalline urea to an aliquot of the sample; H_2O alone was added to bring duplicate aliquots of the sample to the same concentration without urea. Cytoskeletal proteins or control proteins (e.g., monoclonal antibodies) were added to fluorescent samples (AEDANS-A2 or AEDANS-ME) or to controls (dialysis buffer) in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$.

$NaDodSO_4$ Gel Electrophoresis and Other Analytical Techniques. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by using the buffer system of Laemmli (1970) with a linear 7–15% gradient of acrylamide in a 30-cm-long running gel. Samples were prepared by addition of 12.5% trichloroacetic acid to a protein sample (10–150 μg) and incubation for 1–4 h on ice. The precipitated protein was collected by centrifugation, washed 1 time with acetone to remove excess trichloroacetic acid, and dissolved by boiling for 3 min in sample buffer. Fluorescence patterns were photographed prior to staining gels with Coomassie blue, using stain A of Vesterberg & Hansen (1977). For some gels, duplicate lanes were stained by the periodic acid–Schiff (PAS) method. Some stained gels were scanned by a densitometer (Ortec Model 4310). In some cases, fluorescent bands were excised from unfixed gel slices by three 24-h extractions into 1.0 mL of 0.1% $NaDodSO_4$ at 37 °C, and the extracts were pooled.

Stoichiometry of AEDANS incorporation was determined by using [^{3}H]IAEDANS, synthesized by the method of Hudson & Weber (1973). The product was characterized by high-performance liquid chromatography on a Zorbax-C8 4.6 mm × 25 cm column (Du Pont) using a solvent mixture of 25% methanol and 75% 0.1 M sodium acetate–acetic acid, pH 5.0, at a flow rate of 1.5 mL/min; over 80% of the ^{3}H ra-

dioactivity migrated with pure IAEDANS (Sigma). The specific activity of the [^{3}H]AEDANS was measured by liquid scintillation counting in Aquasol and absorbance spectroscopy using an extinction coefficient of 6000 cm^{-1} M^{-1} for AEDANS-ME at 337 nm. The labeling of the HLA-A2 heavy chain (p44) was quantitated by excising and extracting the fluorescent p44 band from a gel, subjecting it to acid hydrolysis, and then determining both the [^{3}H]AEDANS content (by liquid scintillation counting) and the protein content (by quantitative amino acid analysis on an amino acid analyzer) of the hydrolysate. Other protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Fluorescence Spectroscopy. Corrected emission and excitation spectra were recorded by using an Aminco SPF 1000 CS spectrofluorometer with emission and excitation slit widths of 10 nm. Mean fluorescence anisotropy measurements were carried out on the same fluorometer using an excitation wavelength of 350 nm (slit width 10 nm) and an emission wavelength of 490 nm (slit width 20 nm) by measuring all combinations of (excitation, emission) at 0 and 90°. The corrected ratio (R) for each sample was calculated as

$$R = \frac{(0,0)/(0,90)}{(90,0)/(90,90)}$$

and the mean anisotropy ($\bar{A}$) was calculated as

$$\bar{A} = \frac{R-1}{R+2}$$

Uncorrected intensity measurements were routinely performed with a Perkin Elmer 512 spectrofluorometer in the ratio mode, with an excitation wavelength of 350 nm (slit width 10 nm) and an emission wavelength of 490 nm (slit width 10 nm). All measurements were made at room temperature (~23 °C), and the fluorescence of appropriate blank samples was subtracted in all cases.

An Ortec 9200 nanosecond photometer was used to measure the mean emission lifetime (τ). A single τ value was fit to the emission decay data by a least-squares analysis (Grinvald & Steinberg, 1974). The emission decay of quinine sulfate in 1 N H_2SO_4 was also recorded as a test of the system.

Results

Purification of HLA-A2 Antigen. Allospecific monoclonal antibodies have been used for rapid, high-yield purification of HLA-A2 and -B7 antigens solubilized by papain proteolysis (Parham, 1979). When the HLA-A2 and -B7 antigens were solubilized from JY cell lymphoblastoid cell membranes by detergent extraction and subsequently purified by immunoaffinity chromatography, the resultant HLA-A2 and -B7 antigens were "contaminated" with actin. The extent of this contamination was not initially appreciated because actin is not resolved from the detergent-solubilized HLA heavy chain (p44) on a standard gel system (8-cm-long $NaDodSO_4$–12.5% polyacrylamide gels using the discontinuous buffer system of Laemmli). However, actin may be cleanly separated from p44 on a 30-cm-long $NaDodSO_4$–7–15% linear gradient polyacrylamide gel in the same buffer system. The contaminant was identified as actin by its exact comigration with rabbit skeletal muscle actin, its characteristic position on isoelectric focusing, and its lack of staining with PAS (in contrast to HLA heavy chain). The Coomassie blue staining intensity of the actin ranged from 5 to 50% of that of the p44, varying from preparation to preparation. Subsequent rechromatography of the HLA-A2 antigen contaminated by actin on *Lens culinaris* (lentil) lectin–Sepharose did not separate the two proteins; i.e., actin, which is not glycosylated, adheres to the lectin with the HLA antigen and is coeluted with the HLA antigen upon addition of competing sugar. This observation suggested that some HLA-A2 molecules exist in a complex with actin. Further, this complex appeared to resist dissociation by all treatments short of protein denaturation or proteolytic cleavage of the carboxy-terminal region from the HLA heavy chain (data not shown). Because actin is readily labeled by fluorescent alkylating agents, it became essential to prepare HLA-A2 free of actin in order to specifically label the carboxy terminus of the HLA antigen. Furthermore, the presence of actin seriously reduced the efficiency of labeling of the carboxy terminus of the HLA antigens (data not shown).

It was proposed to attack the problem of preparing actin-free HLA antigens by removal of the actin from the cell membranes prior to detergent solubilization of the HLA antigen. The experimental protocol developed to accomplish this is described under Experimental Procedures and the $NaDodSO_4$–polyacrylamide gel analysis of the fractions from this scheme is presented in Figure 1. In brief, a mixture of cytoskeletal proteins (containing greater than 90% of the membrane-associated myosin and greater than 60% of the membrane-associated actin) could be extracted from the lymphoblastoid cell membranes by a buffer containing ATP and DTT and low ionic strength. Nuclear histones, contaminating the membrane preparation, were also removed by this treatment. The stripped membranes were then selectively extracted by Brij detergent, solubilizing a mixture of proteins, greatly enriched for HLA-A, -B, and -DR antigens. The Brij detergent extract was then subjected to immunoaffinity chromatography with stringent washing (1 M Tris-HCl, pH 8.0); this led to bleeding of some HLA-A and -B antigens from the column, but the resultant eluate contained very pure HLA-A and -B antigens. The yield from 50 g of JY cells was about 2 mg of HLA-A2 or -B7 antigens, or about one-half of the yields reported for the less pure preparations (Robb et al., 1976). The few minutes of exposure to high pH (50 mM diethylamine, about pH 11.5, see Experimental Procedures) during elution leads to little permanent denaturation, as greater than 80% of the antigen will rebind to the allospecific monoclonal antibody or to an anti-β_2-microglobulin monoclonal antibody.

Fluorescent Labeling of HLA-A2 Antigens. The concentrated actin-free HLA-A2 antigen solution was labeled by gentle reduction with 2.5 mM DTT and alkylation with 10 mM IAEDANS at pH 8.1. The labeling was restricted to the heavy chain (p44) and induced a small decrease in the $NaDodSO_4$–polyacrylamide gel mobility of p44 (Figure 2), corresponding to an increase in the apparent molecular weight of about 2000. The labeled antigen (AEDANS-A2) was separated from free label by lentil lectin affinity chromatography as described under Experimental Procedures; about half of the labeled protein was recovered in the sugar-eluted fractions (Figure 2). In similar experiments using unmodified HLA-A2, about the same proportion of antigen can be recovered in the sugar eluate; the remainder does not adhere to lentil lectin affinity columns. The failure of a fixed fraction of the total HLA-A2 to adhere to lentil lectin–Sepharose may reflect carbohydrate microheterogeneity since only a small portion of the nonadherent HLA-A2 will adhere to a fresh column. In contrast, most of the adherent fraction will rebind to a second lentil lectin affinity column after dialysis to remove methyl α-mannoside (data not shown).

The labeling of the heavy chain by IAEDANS is confined to the intracellular carboxy-terminal region, as revealed by

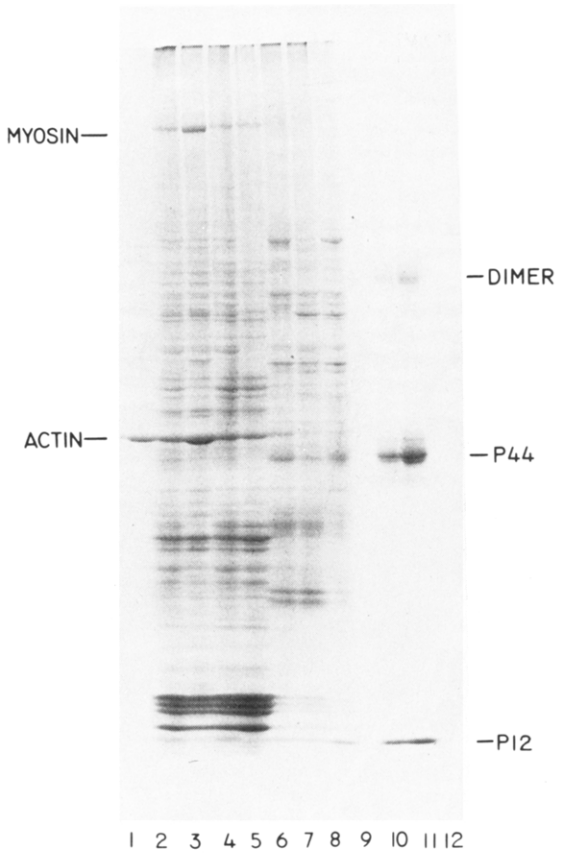

FIGURE 1: Coomassie blue stained $NaDodSO_4$–polyacrylamide gel showing the steps in purification of HLA-A2 antigen, as described under Experimental Procedures. (1) Rabbit skeletal muscle actin (gel standard); (2) JY lymphoblastoid cell membrane pellet; (3) cytoskeletal extracted proteins; (4) residual stripped membranes; (5) Brij detergent insoluble membrane residue; (6) Brij detergent extracted proteins from the stripped membranes; (7) flow through from the immunoaffinity columns; (8) high-salt (1 M Tris-HCl, pH 8.0) wash of the PA2.1–Sepharose column; (9–12) fractions eluted from the PA2.1–Sepharose column with high pH (11.5) solution. Pure HLA-A2 is present as a complex of heavy chain bands (p44) and a light chain band (p12) in lanes 10 and 11. None of the heavy chain bands exactly comigrate with actin, and all are PAS stain positive (which actin is not). This microheterogeneity of the HLA-A2 heavy chain may arise from differences in glycosylation. See Experimental Procedures for experimental details. The tentative identification of a band as myosin is based on $NaDodSO_4$–polyacrylamide gel mobility. Note that approximately 90% of the myosin and 60% of the actin are extracted as cytoskeletal proteins by ATP–DTT–low-salt extraction buffers (lane 3).

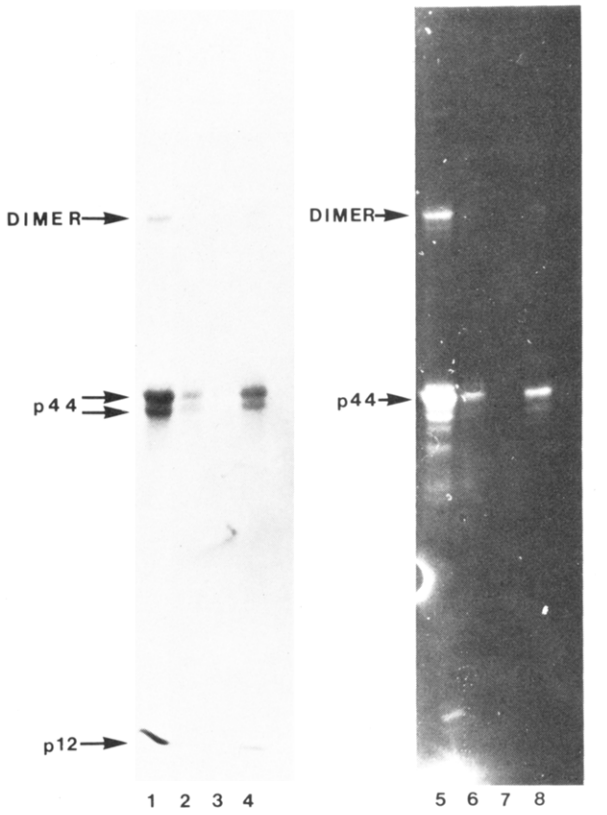

FIGURE 2: Coomassie blue stained $NaDodSO_4$–polyacrylamide gel (lanes 1–4) and the fluorescence pattern of the same gel prior to staining (lanes 5–8) showing the purification of AEDANS-labeled HLA-A2 (AEDANS-A2) by lentil lectin affinity chromatography. (1, 5) Initial labeling mixture of IAEDANS and HLA-A2; (2, 6) flow through of the lentil lectin–Sepharose column; (3, 7) last wash fraction of the same column; (4, 8) proteins eluted by 4% methyl α-mannoside. Note the AEDANS-ME present in lanes 5 and 6 near the gel front. During the reaction of HLA-A2 with IAEDANS, the apparent molecular weight of a portion of the HLA-A2 heavy chain has increased, as shown by the appearance of the upper chain of the doublet. Most of the fluorescence is coincident with the new upper band. (The small amount of fluorescence seen with the lower band of the doublet may represent nonspecific association.)

limited proteolysis. Trypsin releases the carboxy-terminal region from native HLA-A2 (p44,12) to produce a limit product (p39,12). As shown in Figure 3, the conversion of p44 to p39 by trypsin is coincident with the release of AEDANS fluorescence, indicating that the AEDANS is attached to the carboxy-terminal domain. Similar results were observed with proteolysis of HLA-A2 by papain (data not shown). It is interesting that the heavy chain doublet generated during the labeling reaction with IAEDANS appears to persist in the p39,12 product. This observation may suggest that the shift to increased molecular weight of the heavy chain during labeling is caused by a stable conformational change which persists in $NaDodSO_4$ and is not related to incorporation of AEDANS. However, the heavy chain product (p39) of trypsin proteolysis of unmodified HLA heavy chain often shows heterogeneity on high-resolution $NaDodSO_4$–polyacrylamide gel electrophoresis, and the p39 doublet seen in Figure 3 may reflect heterogeneity of the tryptic cleavage products. The carboxy-terminus sequence of the HLA-A2 antigen shows several potential tryptic cleavage sites within a sequence of seven residues (Robb et al., 1978), supporting this hypothesis.

The stoichiometry of AEDANS labeling was determined by use of [^{3}H]-IAEDANS. Two separate labelings gave values of 0.3 and 0.6 mol of AEDANS/mol of HLA-A2, respectively. The relative staining intensity of the fluorescently labeled band to the unlabeled band (seen in Figure 2) is consistent with a stoichiometry of about 0.6 mol of AEDANS/mol of HLA-A2.

Spectral Characteristics of Fluorescently Labeled HLA-A2. AEDANS attached to the carboxy-terminal domain of HLA-A2 (AEDANS-A2) senses a markedly different environment than AEDANS attached to β-mercaptoethanol (AEDANS-ME). This is shown by the 20-nm difference in emission maxima of AEDANS-A2 and AEDANS-ME in 0.25% Brij detergent (Figure 4). The addition of a protein denaturant (8 M urea) causes a 10-nm shift to a higher wavelength of the emission maximum of AEDANS-A2, making it more similar to that of AEDANS-ME. This is accompanied by an increase in the quantum yield. AEDANS-ME also has an increased quantum yield in 8 M urea but changes its emission maximum to a slightly lower wavelength (see Table I).

There is also an effect of urea upon the excitation spectrum of AEDANS-A2 (Figure 5). The excitation spectrum of AEDANS-A2 shows two shoulders at approximately 270 and

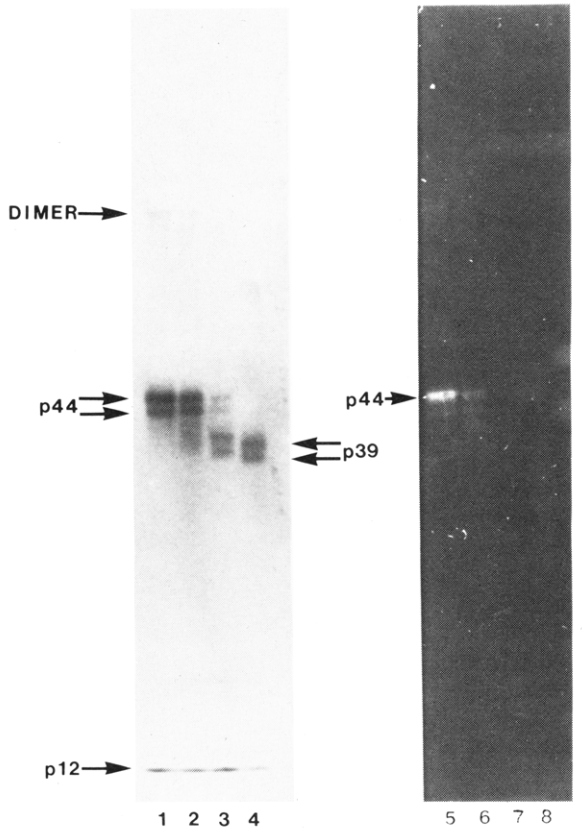

FIGURE 3: Coomassie blue stained $NaDodSO_4$–polyacrylamide gel (lanes 1–4) and the fluorescence pattern of the same gel prior to staining (lanes 5–8) showing the proteolysis of AEDANS-A2 by trypsin. The weight ratio of trypsin to HLA-A2 is 0, 1:200, 1:50, and 1:10 for lanes 1–4 (or 5–8), respectively. Note that as the carboxy-terminal hydrophilic intracellular region is removed (p44 is converted to p39), the AEDANS fluorescence is coincidentally excised.

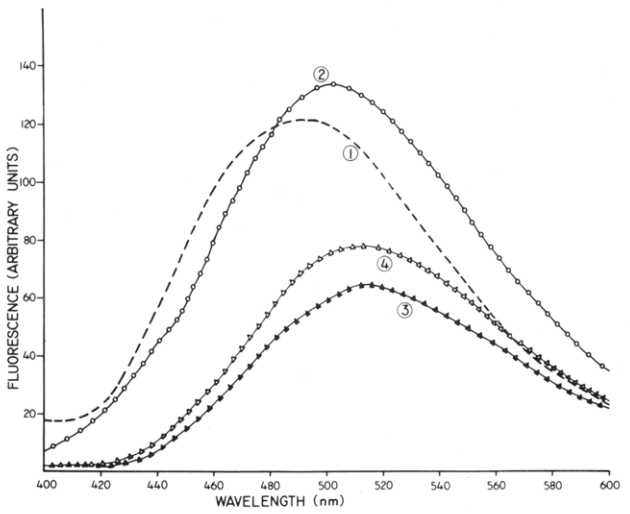

FIGURE 4: Corrected emission spectra of AEDANS-labeled molecules, all samples excited in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$ at 350 nm. Curve 1 is AEDANS-A2 in 0.25% Brij, curve 2 is AEDANS-A2 in 0.25% Brij made 8 M in urea, curve 3 is AEDANS-ME in 0.25% Brij, and curve 4 is AEDANS-ME in 0.25% Brij made 8 M in urea. The concentration of AEDANS is equal for the HLA-A2 samples but is somewhat lower in the two AEDANS-ME samples. Note that the curves for AEDANS-A2 have emission maxima at a lower wavelength than those for AEDANS-ME and that the addition of urea reduces the difference.

285 nm, neither of which is present in the spectrum of AEDANS-ME. These probably represent fluorescence resonance energy transfer in AEDANS-A2 from nearby protein aromatic groups to AEDANS. Upon addition of 8 M urea, these

Table I: Fluorescence Characteristics of AEDANS-Labeled Molecules[a]

samples	mean anisotropy	emission max (nm)
AEDANS-ME in 0.25% Brij	0.001	516
AEDANS-ME in 0.25% Brij, 8 M urea	0.001	513
AEDANS-A2 in 0.25% Brij	0.085	495
AEDANS-A2 in 0.25% Brij, 8 M urea	0.054	504

[a] All solutions are 150 mM NaCl and 10 mM Tris-HCl, pH 8.0. The mean anisotropy ($\bar{A}$) is given by the expression $(R-1)/(R+2)$ where R is the corrected ratio of emission intensity parallel to the exciting light to emission intensity perpendicular to the exciting light. Excitation is at 350 nm; emission is measured at 490 nm.

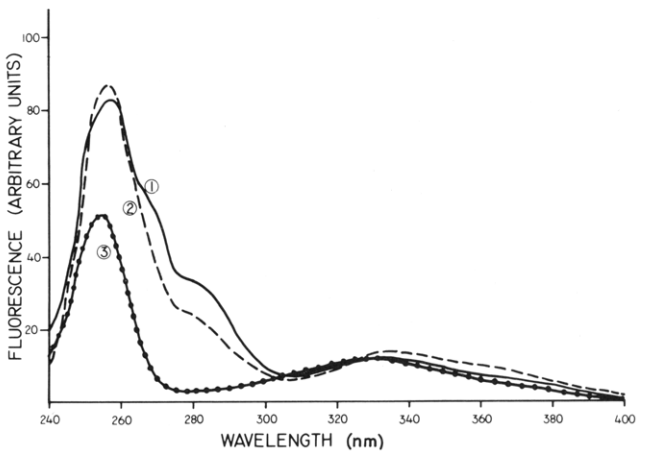

FIGURE 5: Corrected excitation spectra of AEDANS-labeled molecules, all emission observed at 490 nm in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$. Curve 1 is AEDANS-A2 in 0.25% Brij, curve 2 is AEDANS-A2 in 0.25% Brij and 8 M urea, and curve 3 is AEDANS-ME in 0.25% Brij. Both AEDANS-A2 samples are present at an equal concentration whereas AEDANS-ME is present at a somewhat lower concentration. Note that the addition of urea diminishes the shoulders in the aromatic region of curve 1, suggesting loss of energy transfer from protein aromatic groups to AEDANS.

shoulders are markedly diminished. Urea (8 M) has no discernible effect on the excitation spectrum of AEDANS-ME. Thus, urea probably affects the spectrum of AEDANS-A2 by altering the conformation of protein, leading to a geometry that reduces the fluorescence resonance energy transfer and changes the environment of the probe.

Fluorescence polarization measurements of AEDANS-A2 in several environments are reported in Table I. In detergent (0.25% Brij), the mean anisotropy is low, showing that DANS is relatively free to rotationally diffuse. In 8 M urea plus detergent, the mean anisotropy is significantly lower still, again suggesting protein unfolding. In contrast, AEDANS-ME rotates so rapidly that its mean anisotropy is essentially 0 in both detergent (0.25% Brij) and 8 M urea plus detergent. *Thus, the emission spectrum, the excitation spectrum, and the fluorescence polarization measurements all suggest that AEDANS attached to the carboxy-terminal intracellular domain of HLA-A2 senses the conformation of the protein.*

The mean lifetime of the excited state of AEDANS-A2 is longer than that of AEDANS-ME (Table II). However, the decay of fluorescence of AEDANS-A2, in contrast to that of AEDANS-ME, is not well approximated by a single exponential. This suggests either that the labeling of AEDANS-A2 within the carboxy-terminal region is chemically heterogeneous or that AEDANS-A2 can exist in multiple conformational states whose rate of interchange is much slower than the rate

Table II: Mean Fluorescence Lifetime of AEDANS-Labeled Molecules[a]

sample	lifetime (ns)	X^2
AEDANS-ME in 0.1% DOC	9.8	2.9
AEDANS-A2 in 0.1% DOC	12.7	15.5
AEDANS-A2 in 0.1% DOC + cytoskeletal proteins	15.5	4.7

[a] X^2 is a measure of the deviation from a single exponential fit. On the apparatus used, quinine sulfate in 1 N H_2SO_4 had a lifetime of 19.2 ns and an X^2 value of 2.3.

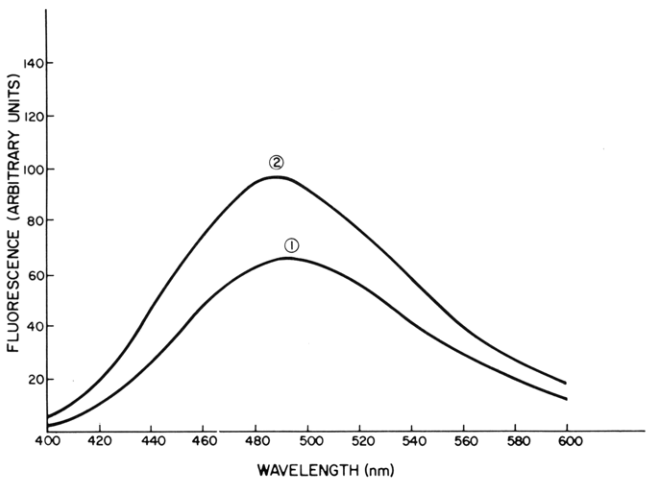

FIGURE 6: Corrected emission spectra of AEDANS-A2 in 0.1% DOC, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$ excited at 350 nm. Curve 1 is the spectrum of AEDANS-A2 alone, curve 2 is the spectrum of AEDANS-A2 upon addition of a saturating amount of the total cytoskeletal extract. The concentration of AEDANS-A2 is equal in both samples. Note that the emission maximum is shifted to a lower wavelength and that the fluorescence intensity is increased upon addition of total cytoskeletal extract. The addition of control proteins (BB7.1 and PA2.1 monoclonal antibodies) to AEDANS-A2 in place of the cytoskeletal extract produced no significant change in the fluorescence emission of the AEDANS reporter group (data not shown).

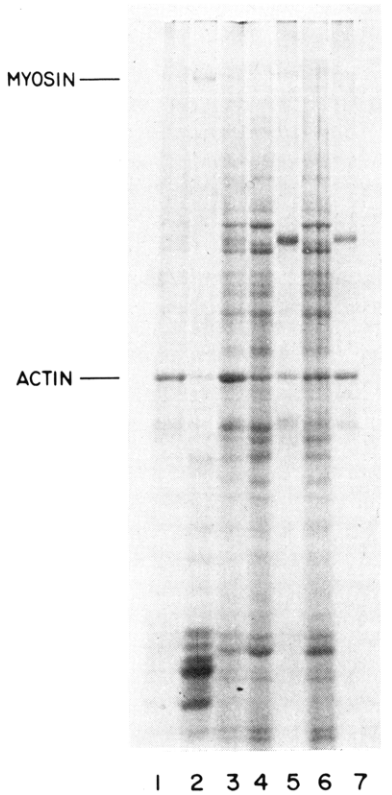

FIGURE 7: Coomassie blue stained NaDodSO$_4$–polyacrylamide gel showing the steps in purifying the cytoskeletal proteins as described under Experimental Procedures. (1) Initial cytoskeletal extract; (2) proteins precipitated by concentration and collected by centrifugation (myosin and histone rich); (3) total soluble extract remaining in solution; (4 and 6) actin-poor supernatants left after low and high KCl (80 and 800 mM, respectively) polymerization and centrifugation; (5 and 7) actin-rich pellets after low and high KCl (80 and 800 mM, respectively) polymerization and centrifugation. Note that actin (45 000 daltons) and at least two other bands (about 36 000 and 80 000 daltons, respectively) are concentrated by the KCl polymerization step.

of decay of the AEDANS excited state.

Interaction of AEDANS-A2 with the Cytoskeleton. During the initial purification of HLA-A2 from lymphoblastoid cell membranes, it was discovered that actin copurified with HLA-A and -B antigens and that this actin appeared to form a stable complex with the purified HLA-A and -B antigens not dissociable on lentil lectin affinity columns. In order to produce actin-free HLA-A2 and -B7, it proved necessary to extract membrane-bound cytoskeletal proteins prior to detergent solubilization of the membranes. It seemed interesting to determine if these cytoskeletal proteins could be recombined with the purified HLA-A2 antigen and if the interaction could be shown to take place through the carboxy-terminal intracellular region of the HLA-A2 antigen. Figure 6 shows that recombination of AEDANS-A2 with a concentrate of the cytoskeletal extract (prepared as described under Experimental Procedures and shown in lane 3 of Figure 7) produced an increase in the quantum yield and a small shift to lower wavelength (5–10 nm) in the fluorescence emission maximum of the AEDANS reporter group. This effect was dependent upon the amount of cytoskeletal extract added and saturated at a 45% increase over the initial fluorescence intensity. The ratio of protein in the cytoskeletal extract to the HLA antigen that produced a half-maximal change in fluorescence intensity was about 2 as determined by Lowry protein assays; this assay overestimates the amount of HLA antigen. The change was also time dependent and reached 90% of the final value within 2 min of mixing. No such change in fluorescence intensity was detected when the cytoskeletal proteins were added to AEDANS-ME. The mean fluorescence lifetime of AEDANS-A2 also increased upon addition of cytoskeletal proteins and became more homogeneous (Table II).

What is the basis of these changes? It is not simply addition of protein since addition of BB7.1, a noninteracting monoclonal antibody, at 5–10-fold higher concentration than that of the protein in the cytoskeletal extract, has no effect upon the fluorescence intensity of AEDANS-A2 (data not shown). It is probably more specific than simple interaction with the HLA-A2 protein because addition of PA2.1, a monoclonal antibody that binds to the extracellular region of HLA-A2, produces only a small (less than 5%), possibly insignificant increase in the fluorescence intensity of AEDANS-A2 even when added at a 5–10-fold higher concentration than that of the protein in the cytoskeletal extract. Thus, the fluorescence changes produced by cytoskeletal proteins are probably caused by specific interactions between the cytoskeletal proteins and the carboxy-terminal region of the HLA-A2 molecule.

When actin and its associated proteins are purified from the mixture of cytoskeletal proteins by polymerization and centrifugal collection of actin polymers (Figure 7), the resultant actin-rich fraction is 3-fold more potent than the residual, partially actin depleted mixture of proteins (measured as the concentration of protein which produces 50% of the

maximal increase in fluorescence intensity). Densitometry of the gel patterns of these two fractions suggests that the actin-rich fraction is 2 times more enriched in actin than the partially actin depleted fraction and has a much simpler protein composition. This evidence is suggestive that actin or some other protein in the actin-rich fraction is responsible for the interaction with HLA-A2 detected by the AEDANS reporter group. Rabbit skeletal muscle actin does not produce the change in the fluorescence signal seen with membrane-bound cytoskeletal proteins. A few proteins other than actin are also enriched in the actin-rich fraction, and each of these is a potential candidate for the cytoskeletal protein which interacts with the HLA-A2 antigen. *Rigorous identification of the relevant cytoskeletal protein(s) awaits subsequent purification of the fluorescence enhancing activity.*

Discussion

The results presented show that detergent-solubilized HLA-A and -B antigens (p44,12) may be purified from lymphoblastoid cell membranes on a milligram scale using immunoaffinity chromatography with allospecific monoclonal antibodies. However, unless membrane–cytoskeletal interactions are disrupted prior to detergent extraction of the HLA-A and -B antigens, the resultant preparations are contaminated by cytoskeletal actin. The amount of actin present is variable and less than stoichiometric. The amount of actin may depend upon the physiologic state of the cell prior to freezing and hypotonic lysis; i.e., the presence of actin may reflect a variable in vivo association between the cytoskeletal and the HLA-A and -B antigens that persists through the purification procedure. Alternatively, the presence of actin in the HLA antigen preparations may reflect an artifactual association produced during purification. In any event, the association appears to be between actin and HLA-A and -B and not mediated by the immunoglobulins used in the purification. This is shown by the facts that no immunoglobulin molecules are detectable in the final preparation and that the actin–HLA-A2 association persists upon lentil lectin chromatography. The extraction of cytoskeletal proteins used in this paper is a novel step in the purification of a plasma membrane protein and may prove useful in other cases as well.

The fluorescent labeling of an HLA antigen in the intracellular carboxy-terminal region as described in this paper may have several experimental uses. First, such antigens are not modified in their extracellular, immunologically important regions and should be readily recognized by specific receptors for HLA antigens. Fluorescein iodoacetamide or eosin iodoacetamide, having much higher extinction coefficients than that of AEDANS, may be successfully substituted in the carboxy-terminal-specific alkylating reaction (unpublished experiments), and such fluorescent HLA antigens may then be used to label (and select) cells bearing HLA receptors. The incorporation of a fluorescent reporter group sensitive to protein conformation in the intracellular portion of a transmembrane protein will also permit studies of transmembrane signaling mechanisms. Our preliminary experiment in this regard, the addition of a monoclonal antibody directed against the extracellular portion of the HLA-A2 molecule (PA2.1) to AEDANS-A2 in detergent solution, produced little significant fluorescence change and served as a negative control for our other experiments. Nevertheless, such a mechanism may exist in biological membranes (albeit not in detergents) and might be studied in liposomes.

Finally, HLA-A and -B antigens, selectively labeled in the intracellular region of the HLA heavy chain, can be used to probe for interactions between membrane proteins and the cytoskeleton. Some successful preliminary results of this line of investigation are presented in this paper. We have found that an HLA-A2 interaction with membrane-derived cytoskeletal proteins may be demonstrated in vitro as detected by signals from the AEDANS reporter group. This interaction appears specific on four grounds: (1) cytoskeletal proteins do not interact with AEDANS-ME, a model compound: (2) an irrelevant, noninteracting antibody (BB7.1) will not produce the effect with AEDANS-A2; (3) an antibody molecule that interacts with the extracellular part of the HLA-A2 antigen (PA2.1) produces a much smaller, possibly insignificant, effect; and (4) the effector molecule appears to copurify with polymerized forms of actin from the cytoskeletal extract. It remains to be determined which molecule of the actin-rich fraction is responsible for the interaction. Once the relevant molecule has been identified and purified, the stoichiometry and binding constant may be determined. It is interesting that mouse lymphocytes appear to have a submembranous molecular scaffolding comprised of a mixture of proteins similar to those in the actin-rich fractions described here (Mescher et al., 1981).

Is the interaction demonstrated here of physiological relevance? The evidence is not yet in. However, data from double immunofluorescence microscopy (Huet et al., 1980; B. C. Guild and J. L. Strominger, unpublished experiments) and from fluorescence photobleach recovery experiments (Petty et al., 1980) strongly suggest that interactions between HLA-A and -B antigens and the cytoskeleton do exist in vivo. Our approach is straightforward: to take the system apart and to recombine the components. The fluorescence reporter group assay is simple and offers an opportunity both to identify proteins which interact with the intracellular region of the HLA heavy chain and to study how these interactions are regulated. For example, does the presence or absence of the phosphate moiety, which is located in the intracellular region of the HLA heavy chain (Pober et al., 1978), have any effect upon the conformationally sensitive fluorescence signal or upon the cytoskeletal interaction? Eventually, HLA-A and -B antigens, fluorescently labeled in the intracellular portion of the heavy chain, may be reinserted into cell membranes by liposome fusion. Such an experiment could test whether the interaction we have demonstrated in vitro occurs in vivo. Furthermore, it would permit membrane protein-cytoskeletal interactions to be studied in vivo as molecular events.

Acknowledgments

We thank Drs. David Golan, James Kaufman, Samuel Latt, Vincent Marchesi, and Matthew Mescher for their and criticism in preparing this manuscript. We also thank Drs. Jonathan Cohen and Samuel Latt for use of their spectrofluorometers.

References

Bourguignon, L. Y. W., Tokuyasu, K. T., & Singer, S. J. (1978) *J. Cell. Physiol. 95*, 239–258.

Engelhard, V. H., Guild, B. C., Helenius, A., Terhorst, C., & Strominger, J. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3230–3234.

Grinvald, A., & Steinberg, I. Z. (1974) *Anal. Biochem. 59*, 583–598.

Hudson, E. N., & Weber, G. (1973) *Biochemistry 12*, 4154–4161.

Huet, C., Ash, J. F., & Singer, S. J. (1980) *Cell (Cambridge, Mass.) 21*, 429–438.

Koch, G. L. E., & Smith, M. J. (1978) *Nature (London) 273*, 274–278.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lux, S. E. (1979) *Nature (London) 281*, 426–429.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
Mescher, M. F., Jose, M. J. L., & Balk, S. P. (1981) *Nature (London)* (in press).
Parham, P. (1979) *J. Biol. Chem. 254*, 8709–8712.
Parham, P., Alpert, B. N., Orr, H. T., & Strominger, J. L. (1977) *J. Biol. Chem. 252*, 7555–7567.
Petty, H. R., Smith, L. M., Fearon, D. T., & McConnell, H. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6587–6591.
Pober, J. S., Guild, B. C., & Strominger, J. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 6002–6006.
Robb, R. J. (1977) Ph.D. Thesis, Harvard University, Cambridge, MA.
Robb, R. J., Strominger, J. L., & Mann, D. L. (1976) *J. Biol. Chem. 251*, 5427–5428.
Robb, R. J., Terhorst, C., & Strominger, J. L. (1978) *J. Biol. Chem. 253*, 5319–5324.
Springer, T. A., & Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2481–2485.
Springer, T. A., Strominger, J. L., & Mann, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1539–1543.
Vesterberg, O., & Hansen, L. (1977) in *Electrofocusing and Isotachophoresis* (Radola, B. J., & Graesslin, D., Eds.) pp 123–133, de Gruyter, Elmsford, NY.

Interaction of Tropomyosin–Troponin with Actin Filaments†

Albrecht Wegner* and Terence P. Walsh

ABSTRACT: The assembly of actin filaments with tropomyosin–troponin was investigated by means of light scattering. Binding curves of tropomyosin–troponin [consisting of all three subunits (holotroponin)] and of tropomyosin–troponin-T–I to actin filaments were analyzed by separating the affinity of tropomyosin–troponin for actin filaments and the affinity for the end-to-end contact of tropomyosin molecules. Under the experimental conditions (42.4 °C, 300 mM KCl), tropomyosin–holotroponin in the absence of calcium and tropomyosin–troponin-T–I had similar affinities for actin filaments whereas tropomyosin–holotroponin in the presence of calcium was found to bind more weakly. Tropomyosin–holotroponin and tropomyosin–troponin-T–I bound about 200–300-fold more strongly to binding sites with adjacent tropomyosin–troponin units than to isolated sites on actin filaments. The equilibrium constant for isolated association with actin filaments was more than 2-fold higher for tropomyosin–holotroponin in the absence of calcium (15 400 M^{-1}) and tropomyosin–troponin-T–I (17 500 M^{-1}) than for tropomyosin–holotroponin in the presence of calcium (6600 M^{-1}). Binding curves of mixtures of tropomyosin–holotroponin in the presence of calcium and of tropomyosin–troponin-T–I were measured and analyzed on the basis of a model of cooperative binding of two types of large ligands to a one-dimensional homogeneous lattice. The results provided information on the strength of the end-to-end contacts of tropomyosin–troponin units in different positions on an actin filament. It was found that a tropomyosin–troponin unit binds adjacently to another unit in a different position on an actin filament about 2-fold more weakly than adjacent to a unit in the same position. With the aid of these results, it was possible to obtain information on the equilibrium distribution of tropomyosin–troponin in the two positions on actin filaments. Generation of a sequence of tropomyosin–troponin units in a different position on actin filaments was found to be 4-fold less favored than elongation of an existing sequence (cooperativity parameter $\sigma = {}^1/_4$). Shifting of tropomyosin–troponin on actin filaments appears to be accompanied by small free-energy changes in the various interactions of the components of actin–tropomyosin–troponin filaments and not to be an all-or-none reaction.

The regulatory role of the tropomyosin–troponin complex in muscle contraction was first described by Ebashi (1963), who showed that at low calcium concentrations the tropomyosin–troponin complex prevents the interaction of myosin with actin whereas at high calcium concentrations actin–tropomyosin–troponin filaments activate ATP hydrolysis by myosin. X-ray and electron microscopic investigations have provided evidence that tropomyosin is shifted from the periphery to the groove of actin filaments on binding of calcium to troponin (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973; Wakabayashi et al., 1975; Seymour & O'Brien, 1980). The inhibition of myosin ATPase in the absence of calcium has been interpreted as a steric blocking of the myosin binding sites on actin filaments by tropomyosin (Huxley, 1972).

Troponin is composed of three nonidentical subunits (holotroponin), the molar ratios of which are still being discussed (Greaser & Gergely, 1971; Sperling et al., 1979). The three troponin subunits are designated troponin-T, the tropomyosin-binding subunit, troponin-I, which is necessary to inhibit the myosin ATPase, and troponin-C, which binds calcium. The tropomyosin–troponin-T–I complex inhibits the actomyosin ATPase independently of the calcium concentration (Greaser et al., 1972), and electron microscopic studies indicate that in actin–tropomyosin–troponin-T–I filaments the tropomyosin is located at the periphery of the actin filaments (Wakabayashi et al., 1975).

Myosin free of ATP binds to actin–tropomyosin–troponin filaments even in the absence of calcium (Bremel & Weber, 1972). Under these conditions, troponin has greater affinity

† From the University of Pennsylvania, School of Medicine, Department of Biochemistry and Biophysics, Philadelphia, Pennsylvania 19174. *Received January 27, 1981.* This study was supported by National Institutes of Health Grants HL 15835 and 15692.

* Address correspondence to this author at the Biozentrum, Abteilung Biophysikalische Chemie, CH-4056 Basel, Switzerland. A.W. received a fellowship from the Deutsche Forschungsgemeinschaft.

0006-2960/81/0420-5633$01.25/0