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Binding Motifs of Copolymer 1 to Multiple Sclerosis- and Rheumatoid Arthritis-Associated HLA-DR Molecules[1]

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Copolymer 1 (Cop 1, poly (Y, E, A, K)) is a random synthetic amino acid copolymer effective in the treatment of relapsing forms of multiple sclerosis (MS). Cop 1 binds promiscuously, with high affinity and in a peptide-specific manner to purified MS-associated HLA-DR2 (DRB1*1501) and rheumatoid arthritis-associated HLA-DR1 (DRB1*0101) or HLA-DR4 (DRB1*0401) molecules. In the present work at least 95% of added Cop 1 could be bound to recombinant "empty" HLA-DR1 and -DR4, and 80% could be bound to HLA-DR2 proteins. Amino acid composition, HPLC profiles, and sequencing patterns of Cop 1 eluted by acid extraction from HLA-DR molecules were similar to those of the unseparated Cop 1. Protruding N-terminal ends of Cop 1 bound to HLA-DR1, -DR2, or -DR4 molecules were then treated with aminopeptidase I, followed by elution, HPLC, and pool sequencing. In contrast to untreated or unbound Cop 1, this material exhibited distinct motifs at some positions with increases in levels of E at the first and second cycles, of K at the second and third cycles, and of Y (presumably at P1 of the bound peptide) at the third to fifth cycles, regardless of the HLA-DR molecule employed. No preference was seen at the following cycles that were mainly A. These first pooled HLA-DR binding epitopes provide clues to the components of Cop 1 that are biologically active in suppressing MS and possibly rheumatoid arthritis. *The Journal of Immunology,* **1999, 162: 4697–4704.**

Copolymer 1 (Cop 1[3], poly (Y, E, A, and K)) is a synthetic amino acid copolymer effective both in suppression of experimental allergic encephalomyelitis (EAE) (1–12) and in the treatment of relapsing forms of multiple sclerosis (MS) (13, 14). The mechanisms proposed for the activity of Cop 1 involve binding to class II MHC molecules on APCs (9), leading to induction of Ag-specific suppressor cells (4, 6) and/or competition with myelin Ags for activation of specific effector T cells (7, 8). Indeed, the binding of Cop 1 to purified human HLA-DR molecules within the peptide binding groove has been reported (15). Cop 1 inhibited the binding of HA 306–318 peptide, a high-affinity epitope of influenza virus, to both HLA-DR1 (DRB1*0101) and -DR4 (DRB1*0401) molecules, and of myelin basic protein (MBP) 84–102, a human immunodominant epitope of MBP, to HLA-DR2 (DRB1*1501) molecules (15). Moreover, Cop 1 has been recently found to compete with collagen type II (CII) 261–273, a candidate autoantigen in rheumatoid arthritis (RA), for binding to RA-associated HLA-DR1 (DRB1*0101) and -DR4 (DRB1*0401) molecules, and to inhibit CII-reactive T cell clones (16). The characterization of the active component(s) of the mixture of random polypeptides has thus been of particular importance in view of the therapeutic applications of Cop 1 in MS and possibly RA patients.

Because Cop 1 is a mixture of random polypeptides, it may contain different sequences that bind to different HLA proteins; in this case only a fraction out of the whole mixture would be an "active component." Alternatively, the whole mixture may be competent, i.e., all polypeptides binding to any HLA-DR molecule. In view of the crystallographic analysis of several HLA-DR molecules complexed with the antigenic peptides (17–19), as well as the binding motifs of natural MHC-associated ligands that were elucidated by acid-extraction and sequencing (20–25), it has been intriguing to find out whether all four amino acids that compose Cop 1 are involved in its binding in the groove of HLA-DR molecules.

The study here was undertaken to attempt to identify these active components present in the bound Cop 1 and to determine their binding motifs. To isolate the bound fraction of Cop 1 with no interference from endogenous peptides, recombinant "empty" HLA-DR1, -DR2, and -DR4 molecules produced in insect cells were employed. Because the average length of the Cop 1 polypeptides used is 75–80 amino acids, the epitopes lying in the groove of HLA-DR molecules are likely to be found internally in the polypeptide chains with protruding ends, making direct analysis of the bound amino acid sequences by microchemical methods very complicated. To access these regions, N-terminal peptidase treatment of the protruding ends of Cop 1 polypeptides was employed. This approach proved to be useful in trimming of N-terminal ends of peptides that protrude out of class II MHC proteins, while protecting epitopes bound to the groove from proteolysis (26, 27). Various characteristics relating to the fraction bound and its motifs, including amino acid composition, HPLC profiles, and pool sequencing, together with the immunological recognition of these fractions, are presented here.

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[3] Abbreviations used in this paper: Cop 1, copolymer 1; CII, collagen type II; HA, influenza virus hemagglutinin; MBP, myelin basic protein; MS, multiple sclerosis; RA, rheumatoid arthritis; EAE, experimental allergic encephalomyelitis; RP, reverse phase.

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Materials and Methods

Protein expression and purification

Soluble HLA-DR1, -DR2, and -DR4 molecules were expressed in Drosophila S2 cells as described (18, 28, 29). Cells were grown in roller bottles in ExCell 401 medium (JRH Biosciences, Lenexa, KS) supplemented with 0–5% fetal bovine serum (Sigma Chemicals, St. Louis, MO) at 26°C. Cells were harvested 4–5 days after induction by 1 mM $CuSO_4$. Immunoaffinity purification of HLA-DR1, -DR2, and -DR4 molecules was performed as reported previously (18). Briefly, supernatant from harvested cells was sequentially passed through protein A, protein G, and protein A-LB3.1 columns, followed by elution of the bound HLA-DR with 50 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 11.5) and neutralized with 200 mM phosphate (pH 6.0). Proteins were concentrated on a Centriprep 10 membrane (Amicon, Beverly, MA).

Antigens and Antibodies

Cop 1 is a synthetic random copolymer prepared by polymerization of the *N*-carboxyanhydrides of L-tyrosine, γ-benzyl-L-glutamate, L-alanine, and ϵ,*N*-trifluoroacetyl-L-lysine (1) (the end product is a mixture of acetate salts of random polypeptides). Cop 1, batch 52596, in the molar ratio of 1 Y:1.5 E:4.3 A:3.3 K, with an average m.w. of 8150, was obtained from Teva Pharmaceutical Industries (Petach Tikva, Israel). Rabbit anti-Cop 1 polyclonal Abs (IgG fraction, biotin-labeled) were also from Teva Pharmaceutical Industries.

Treatment of HLA-DR-Cop 1 complexes with aminopeptidase I

Cop 1 (1 mM) was incubated with recombinant water-soluble "empty" HLA-DR1, -DR2, or -DR4 molecules (100 μM) in PBS for 40 h at 37°C. Aminopeptidase I (2 units) (Sigma Chemicals) was added for the last 18 h of incubation. Samples were then spin-concentrated to a final volume of ~100 μl using Centricon 10 ultrafiltration devices (Beverly, MA). Bound Cop 1 was eluted from HLA-DR by addition of acetic acid (10%) and incubated at 70°C for 15 min, followed by ultrafiltration and vacuum concentration in a SpeedVac (Savant), as described previously (12).

HPLC separation and microsequencing

After elution as above, typically 5–10% of the Cop 1 mixtures were fractionated by microbore HPLC using a Zorbax C_{18} 1.0-mm reverse-phase (RP) column (Saratoga, CA) on a Hewlett-Packard 1090 HPLC with 1040 diode array detector. At a flow rate of 54 μl/min, Cop 1 was eluted with a gradient of 0.055% trifluoroacetic acid in acetonitrile (0% at 0–10 min, 33% at 73 min, and 60% at 105 min). Strategies for peak selection, RP separation, and Edman microsequencing have been described previously (22, 30). Pooled fractions were submitted to automated Edman degradation on a Hewlett-Packard G1005A protein sequencer using the manufacturer's Routine 3.5 analytical method.

PAGE

SDS-PAGE was conducted with the NOVEX (San Diego, CA) minicell electrophoresis system. Separation gel was 10% in acrylamide and stacking gel was 5%. HLA-DR1-Cop 1 complexes were run under nonreducing conditions for 1 h at 200 V, stained with Coomassie Brilliant blue, fixed for 3 h in 10% methanol/10% acetic acid, and dried on Cellophane paper (Bio-Rad, Richmond, CA) at 25°C.

Ab binding assay

The cross-reactivity between Cop 1 and its fractions was detected by direct ELISA assay using biotinylated anti-Cop 1 polyclonal Abs. Cop 1 or fractions were diluted to 0.4 and 2.0 μg/ml and plated in duplicate on a 96-well microtiter immunoassay plates (PRO-BIND, Falcon, Lincoln Park, NJ) (100 μl per well). All incubations were for 1 h at 37°C and washes were three times with Tris-buffered saline (TBS)/0.05% Tween-20 (TBS = 137 mM sodium chloride, 25 mM Tris (pH 8.0), 2.7 mM potassium chloride). The wells were then blocked with TBS/3% BSA, followed by addition of biotinylated anti-Cop 1 Abs (1:5000, 100 μl per well). Ab-ligand complexes were detected using streptavidin-conjugated alkaline phosphatase (1:3000, Bio-Rad) and *p*-nitrophenyl phosphate in triethanolamine buffer (Bio-Rad). The absorbance at 410 nm was monitored by a microplate reader (Dynatech MR4000).

Table I. *Amino acid composition of Cop 1 bound to and eluted from recombinant HLA-DR1, -DR1 and -DR4 molecules*

Cop 1[a]	Amount[b]	Y	E	A	K
Untreated	22.3	0.8	1.7	4.3	3.2
Eluted from DR1	21.5 (96)	0.8	1.3	4.3	4.3[c]
Eluted from DR2	17.8 (80)	1.0	2.0	4.3	4.0[c]
Eluted from DR4	21.7 (97)	0.8	1.1	4.3	5.7[c]

[a] Cop 1 (8150) either incubated with recombinant HLA-DR1, -DR2, and -DR4 molecules and eluted by acid extraction, or untreated, was subjected to amino acid analysis as described in *Materials and Methods*. Values represent molar ratios of amino acids and were calculated with reference to A, whose yields are not reduced by the hydrolysis process.

[b] Nanomole copolymer in the sample, determined by amino acid analysis after acid extraction. Percent binding is shown in parentheses.

[c] Higher levels of K may be due to a contamination with isophenylthiourea. This interpretation is compatible with the observation that no such increase was observed in the more precise pool sequencing data in Fig. 2.

Results

Isolation of the bound fraction of Cop 1

Quantitation and amino acid analysis of Cop 1 bound to HLA-DR1, -DR2, and -DR4 molecules. Cop 1 was incubated with water-soluble HLA-DR1, -DR2, or -DR4 molecules at the molar ratio of 1:1 for 40 h at 37°C. These recombinant "empty" HLA-DR molecules are usually stably assembled in the presence of exogenously added peptide Ag. However, Cop 1 can substitute for peptides in promoting stabilization and with no interference from endogenous peptides (15). Unbound Cop 1 was separated from bound Cop 1 by Centricon ultrafiltration. Bound Cop 1 was then extracted from the HLA-DR by acid treatment (22) and subjected to amino acid analysis. At least 95% of added Cop 1 was bound to isolated HLA-DR1 and -DR4, and 80% was bound to HLA-DR2 proteins (Table I). Cop 1 eluted from HLA-DR1, -DR2, and -DR4 molecules showed ratios of Y:E:A:K similar to unseparated Cop 1 (Table I). These results indicate that the bound fraction of Cop 1 reflects the amino acid composition of the whole mixture and suggest that there is little or no preferential binding of Cop 1 components to different HLA-DR proteins. These data were supported by a different set of experiments in which Cop 1 was incubated with an excess of HLA-DR1, -DR2, and -DR4 molecules that had been purified from human homozygous EBV-transformed B cell lines, and then passed through a size-exclusion column. Nearly all of the Cop 1 was found in the fractions corresponding to the high m.w. complexes with each of the HLA-DR molecules (data not shown), with <10% in each case at the lower m.w. position of Cop 1, also indicating that most of the copolymer binds to these molecules.

HPLC separation. To further characterize the bound fraction of Cop 1 by means of hydrophobicity and size, samples were separated on RP-HPLC (Fig. 1) using an acetonitrile gradient, as described in *Materials and Methods*. In contrast to typical profiles of single peptides or peptide pools (22, 31–34), untreated Cop 1 showed a very broad peak with several smaller peaks, which spread between ~40- and 75-min elution time (Fig. 1*A*). This elution profile is characteristic of a mixture of random polypeptides and resembles HPLC separations of other batches of Cop 1 (unpublished data). Similar profiles were obtained when Cop 1 was eluted from HLA-DR1 (Fig. 1*B*), -DR2 (Fig. 1*C*), or -DR4 (Fig. 1*D*) molecules, suggesting that the bound fraction is similar to the whole Cop 1 mixture in its chemical properties.

Pool sequencing. To analyze the sequence of Cop 1 bound to HLA-DR1, -DR2, and -DR4 molecules, HPLC fractions within the area described in the previous section, were pooled and sequenced. In all cases, the four amino acids of Cop 1 showed random patterns, with significantly higher levels of A over E, Y, and K (Fig.

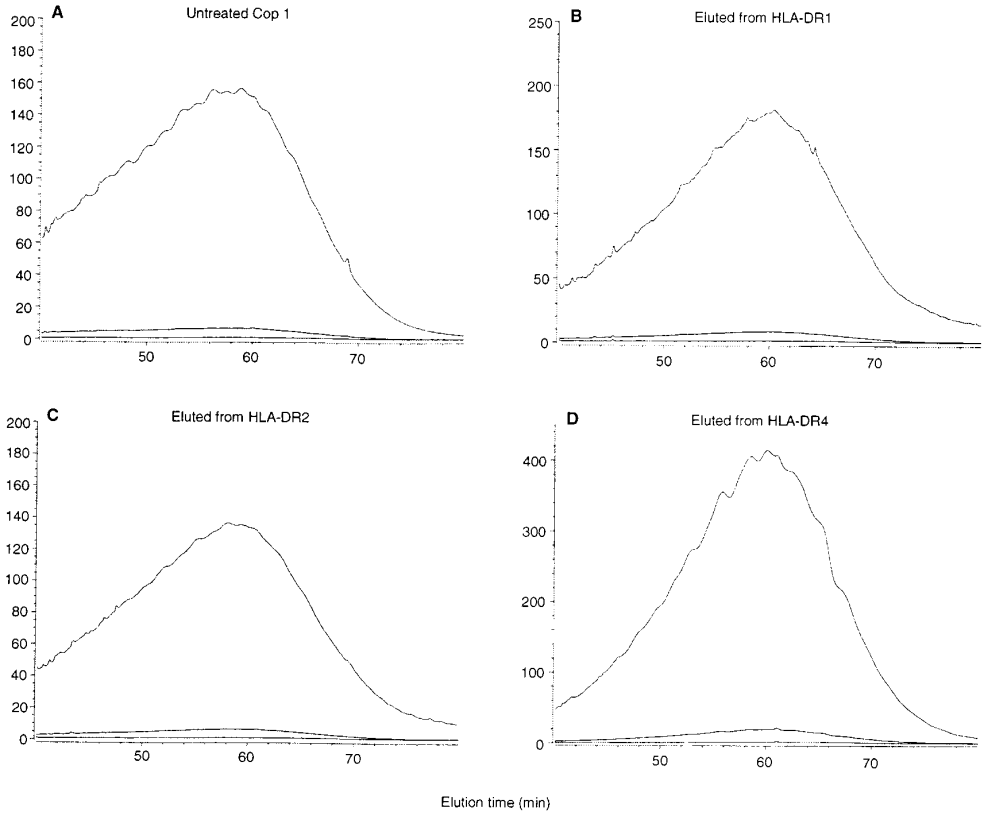

FIGURE 1. Separation of untreated Cop 1 (*A*), Cop 1 eluted from HLA-DR1 (*B*), -DR2 (*C*), and -DR4 (*D*) molecules on RP-HPLC. From 5% to 10% of the protein mixtures were fractionated by microbore HPLC using a Zorbax C_{18} 1.0-mm RP column on a Hewlett-Packard 1090 HPLC with 1040 diode array detector. At a flow rate of 54 μl/min, Cop 1 was eluted with a gradient of 0.055% trifluoroacetic acid in acetonitrile (0% at 0–10 min, 33% at 73 min, and 60% at 105 min). Upper solid line, absorbance at 205 nm; lower solid lines, absorbance at 277 and 292 nm.

2, *A–D*), which corresponds to the initially higher molar ratio of A in Cop 1 (1). There was no sequence specificity or preferential positioning of any amino acid of Cop 1, indicating that the bound fraction is also random, similar to the entire Cop 1. The yields of other amino acids resulting from endogenous peptides of HLA-DR molecules were minor (data not shown). It should be noted that these data represent sequencing of the first 20–25 amino acids from the N termini of bound Cop 1 polypeptides, which most likely protrude from the groove of HLA-DR molecules, and therefore do not reflect the actual binding motif(s).

Recognition of bound Cop 1 by anti-Cop 1 Abs. Anti-Cop 1 polyclonal Abs were used to determine whether fractions of Cop 1 eluted from different HLA-DR molecules contain epitopes similar to unseparated Cop 1. Binding assays were conducted as described in *Materials and Methods.* The results (Fig. 3) showed that all the fractions were similarly recognized by anti-Cop 1 Abs, suggesting that these fractions share similar epitopes with each other and with Cop 1.

Characterization of binding motifs of Cop 1

Treatment of Cop 1 bound to HLA-DR1, -DR2, or -DR4 molecules with aminopeptidase I. To determine the actual binding motifs, Cop 1 was incubated with HLA-DR molecules at the molar ratio of 10 Cop 1:1 HLA-DR in PBS for 40 h at 37°C. Aminopeptidase I, a metalloprotein isolated from *Streptomyces griseus* (35), was added for the last 18 h of incubation, to remove N-terminal ends of Cop 1 polypeptides, protruding from the groove of the HLA-DR molecules, as well as to digest the unbound Cop 1. The resulting Cop 1-HLA-DR complexes were analyzed by SDS-PAGE. As shown in Fig. 4, Cop 1-DR1 complexes were resistant to SDS-induced dissociation, forming higher m.w. complexes with HLA-DR1 $\alpha\beta$ heterodimers that were observed as numerous bands on the polyacrylamide gel above the molecular mass protein standard of 50 kDa (*lane 5*), and as described previously (15). In the presence of aminopeptidase I (a 33-kDa protein appearing as a thin band below the molecular mass protein standard of 35 kDa, *lanes 2*, *4*, and *6*), all the unbound Cop 1 (a smear in the lower part of the gel, *lanes 1* and *5*) was completely digested (*lanes 2* and *6*), whereas Cop 1-DR1 complexes were protected (*lane 6*). It should be noted that upon incubation of aminopeptidase I with HLA-DR1 alone a complex was formed, represented by a band below the 50-kDa molecular mass protein standard (*lane 4*) probably caused by binding of some aminopeptidase I-derived digestion products to HLA-DR1. Similar treatment with aminopeptidase I was applied to Cop 1 bound to HLA-DR2 and -DR4 molecules. Bound Cop 1 remaining after aminopeptidase I treatment was eluted from HLA-DR by acid extraction, as described in *Materials and Methods.*

HPLC separation. After elution, Cop 1 digestion products were separated on RP-HPLC (Fig. 5) using an acetonitrile gradient, as described in *Materials and Methods*. In contrast to a very broad peak corresponding to untreated Cop 1 (Figs. 2*A* and 5*A*), proteolytic Cop 1 fragments eluted from HLA-DR1 (Fig. 5*C*), -DR2 (Fig. 5*E*) or -DR4 (Fig. 5*G*) showed profiles similar to peptide pools

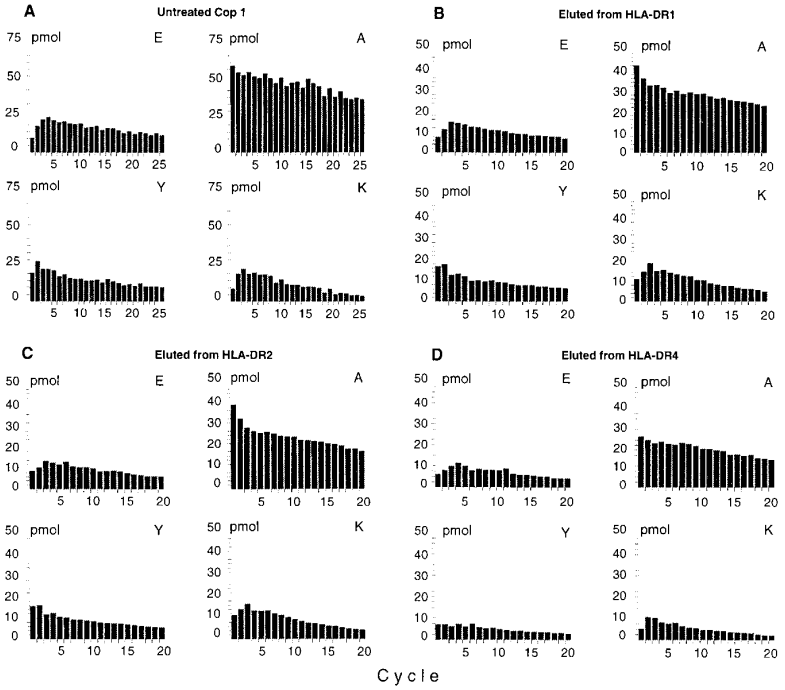

FIGURE 2. Pool sequencing of untreated Cop 1 (*A*), and Cop 1 eluted from HLA-DR1 (*B*), -DR2 (*C*), and -DR4 (*D*) molecules. HPLC fractions were pooled, concentrated, and submitted to automated Edman degradation on a Hewlett-Packard G1005A protein sequencer using the manufacturer's Routine 3.5.

isolated from purified human HLA-DR molecules (31–33). On the other hand, almost no peptides were eluted from "empty" HLA-DR1 (Fig. 5*D*), -DR2 (Fig. 5*F*), or -DR4 (Fig. 5*H*) molecules, or from a total Cop 1 digestion with no HLA-DR added (Fig. 5*B*). Peaks for further analysis were selected in the region where the untreated Cop 1 was eluted (Figs. 2*A* and 5*A*), between ~40 and 75 min elution time. For each HLA-DR molecule, only peaks corresponding to Cop 1 peptides, which did not overlap those eluted from that same HLA-DR molecule with no Cop 1, were pooled for sequencing.

Pool sequencing. To analyze the sequence of the Cop 1 bound to HLA-DR1, -DR2, and -DR4 molecules, Cop 1 fractions were pooled and sequenced. In contrast to random patterns of the untreated Cop 1, showing no sequence specificity or preferential positioning of any of the four amino acids that comprise Cop 1 (Figs. 3*A* and 6*A*), significantly higher levels of E were found at the first and second cycles, of K at the second and third cycles, and of Y (presumably at P1 of the bound peptide) at the third to fifth cycle, for peptides bound to HLA-DR1 (Fig. 6*C*), -DR2 (Fig. 6*E*), or -DR4 (Fig. 6*G*). This spread of residues over two or three positions in the pool sequencing data is probably caused by the ragged N termini of the Cop 1 components after aminopeptidase treatment, similarly to other treated naturally processed class II MHC ligands

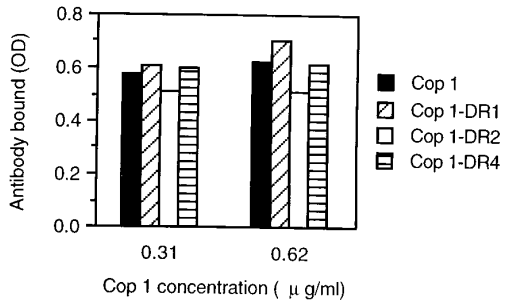

FIGURE 3. Detection of Cop 1 bound to HLA-DR1, -DR2, and -DR4 molecules by anti-Cop 1 Abs. Bound fractions were diluted and plated in duplicates on a 96-well microtiter plates, followed by blocking with TBS/3% BSA and addition of biotinylated anti-Cop 1 polyclonal Abs. For other details see *Materials and Methods*. Background levels were <10% of the binding.

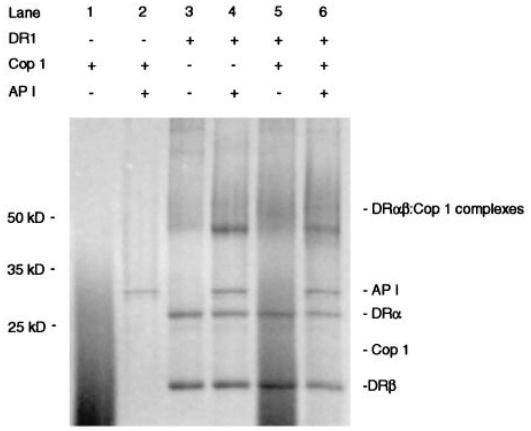

FIGURE 4. SDS-PAGE of soluble Cop 1-DR1 complexes treated with aminopeptidase I. Recombinant "empty" HLA-DR1 molecules (100 μM per sample) were incubated with unlabeled Cop 1 (8, 150) (1 mM) in PBS for 40 h at 37°C. Aminopeptidase I (2 units) was added for the last 18 h of incubation. Cop 1 alone (*lane 1*), or treated with aminopeptidase I (*lane 2*); HLA-DR1 alone (*lane 3*), or treated with aminopeptidase I (*lane 4*); and HLA-DR1-Cop 1 complexes alone (*lane 5*), or treated with aminopeptidase I (*lane 6*). Separation gel was 10% in acrylamide and stacking gel was 5%. HLA-DR1-Cop 1 complexes were run under nonreducing conditions for 1 h at 200 V and stained with Coomassie Brilliant blue.

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