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Crystallization of HLA-DR antigens

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SUMMARY

The class II major histocompatibility antigens HLA-DR1, DR2, DR3, DR4, DR7 and DR8 were purified by immunoaffinity chromatography from homozygous human B lymphoblastoid cell lines. The purified, detergent-soluble molecules were cleaved with the protease papain to remove the hydrophobic transmembrane regions and cytoplasmic tails. Crystals were obtained for each of the papain-solubilized fragments. DR1 crystallized under a variety of different conditions, resulting in two different orthorhombic crystal forms, one of which diffracts as far as 3.5Å. Crystals of DR2, DR3, DR4 and DR8 have the same unit cell dimensions as the DR1 crystals, and crystals of DR3 and DR4 have the same diffracting power as the DR1 crystals. The best DR7 crystals obtained thus far are hexagonal and diffract to only about 8Å. Crystals of similar hexagonal form have also been observed for most of the other DR subsets.

Key-words: MHC, HLA-DR; Class II antigens, Crystals.

INTRODUCTION

The mechanism by which class II major histocompatibility antigens bind and present foreign antigens to T-cell receptors has been the subject of intense investigation for several years. Limited structural information is available for the class II antigens (Kaufman *et al.*, 1984; Gorga *et al.*, 1989; Dornmair *et al.*, 1989) and a model for the structure of the peptide-binding region of class II antigens has been proposed (Brown *et al.*, 1988) based on the three-dimensional structure determined from crystals of water-soluble fragments of the class I molecule HLA-A2 (Bjorkman *et al.*, 1987a, b). Here we report the large-scale preparation and crystallization of water-soluble fragments of HLA-DR1, DR2, DR3, DR4, DR7 and DR8, which should allow the determination of a three-dimensional structure for the extracellular portion of HLA-DR.

MATERIALS AND METHODS

Cell lines

Class II antigens were obtained from Epstein-Barr virus-transformed human B lymphocyte lines: DR1 from LG-2 cells, DR2 from MST cells, DR3 from WT20 cells, DR4 from Priess cells, DR7 from Mann cells and DR8 from 23.1 cells. The cells were grown in roller bottles or spinner flasks in RPMI-1640 medium supplemented with 10 % foetal bovine serum, 2 mM glutamine, 50 units/ml penicillin G and 50 μg/ml streptomycin. The cells were harvested and stored as pellets at −80°C.

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Immunoaffinity purification

Cells (approximately 200 g per preparation) were hypotonically lysed, and a crude membrane fraction was prepared by differential centrifugation, as described in detail previously (Gorga *et al.*, 1987). The membranes were solubilized with Nonidet P-40, and the soluble fraction was passed through a series of immunoaffinity columns in order to isolate the class I and class II antigens of interest. DR was isolated from each cell line using the monoclonal antibody LB3.1 (Gorga *et al.*, 1986) covalently cross-linked to protein A-Sepharose (Schneider *et al.*, 1982). The detergent extracts from Priess and Mann cells were passed through an immunoaffinity column made with the monoclonal antibody 109d6 (Matsuyama *et al.*, 1986), kindly provided by Dr. Robert Winchester, to remove DRw53 prior to passage through the LB3.1 column, since LB3.1 recognizes both DR subsets. DRw52 was not removed, and therefore was likely present as a contaminant in the DR3 preparations. Similarly, the two HLA-DR2 isotypes DR2a and DR2b were not separated. Detergent-soluble HLA-A2 was isolated from the cell line LG-2 using the monoclonal antibody PA2.1 (Parham and Bodmer, 1978) cross-linked to AffiGel 10 (BioRad). The antigens were eluted from the immunoaffinity columns with 50 mM glycine/0.1 % deoxycholate, pH 11.5, and neutralized and dialysed immediately. Protein concentration was determined by BCA assay (Pierce Chemical Co.). Electrophoresis was performed according to the method of Laemmli (1970). Deoxycholic acid was recrystallized from ethanol.

Papain digestion

Water-soluble HLA-A2 was prepared by papain digestion of isolated JY or LG-2 cell membranes, as previously described (Parham *et al.*, 1977). The procedure for papain digestion of class II antigens was adapted from the procedure used previously (Gorga *et al.*, 1987) and was based on the procedure described by Kaufman and Strominger (1979). Papain was activated at 1 mg/ml in 1 mM Na_2EDTA/1 mM dithiothreitol/10 mM tris-(hydroxymethyl)aminomethane (Tris), pH 8.0 (activation buffer) for 5 min at 37°C and diluted to 0.5 mg/ml with activation buffer. Immunoaffinity-purified DR at 1.5 mg/ml in 10 mM Tris/0.1 % deoxycholate pH 8.0 was incubated with 0.375 volume of activated papain at 37°C. The optimum length of digestion with papain was determined for each preparation of immunoaffinity-purified DR using small-scale test digestions. Usually, digestion was complete after 60 min; occasionally, a second aliquot of papain was added after 60 min and the digestion was continued for another 5 to 20 min. The digestion was stopped by the addition of 0.3 volumes of 200 mM iodoacetamide. The mixture was placed on ice and all remaining steps were carried out at 4°C.

Gel filtration

The papain-digested sample was immediately loaded onto a 5.0×90 cm Sephacryl S-200 HR (Pharmacia) column equilibrated with either 10 mM Tris/140 mM NaCl/0.05 % NaN_3 pH 8 or 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES)/0.05 % NaN_3 pH 7.5 at 4°C. Fractions (12.5 ml) were collected at a flow rate of 100 ml/h. Fractions were monitored for absorbance at 280 nm, and papain-solubilized DR was located by SDS-PAGE. Fractions containing solubilized DR were combined, concentrated approximately 10-fold by ultrafiltration on an Amicon PM-30 membrane, and loaded onto a 2.6×90 cm Sephadex G-150 (Pharmacia) column equilibrated with either Tris or HEPES buffer as above. Fractions (3.75 ml) were collected at a flow rate of 8 ml/h. Absorbance at 280 nm was monitored, and fractions containing papain-solubilized DR were confirmed by SDS-PAGE, combined, and concentrated by vacuum dialysis against Tris or HEPES buffer to approximately 10 mg/ml.

Gel filtration analysis of detergent- and water-soluble forms of DR1 and HLA-A2 was performed at room temperature on a 1.0×90-cm BioGel A5m (BioRad) column equilibrated with 10 mM Tris, pH 8.0, with or without 1 % (wt/vol) sodium deoxycholate. Mixtures of protein standards were analysed in the same buffer with and without 1 % (wt/vol) sodium deoxycholate. Peak positions were determined by reading the absorbance at 280 nm and by SDS-PAGE.

Crystallization

Crystals were grown by the vapor-diffusion method in 2-8 μl drops on siliconized glass microscope cover slips inverted over precipitant solutions in 24-well tissue culture plates and sealed with vacuum grease. Initial protein concentrations were between 4 and 20 mg/ml. For each DR allele, a wide range of crystallization conditions was tested, including the use of the "factorial solution" approach suggested by Carter *et al.* (1988). DR crystals were harvested in well solution and mounted in 2-mm diameter glass capillaries. Space groups were determined at room temperature on film using a Supper precession camera and an Elliott GX-6 X-ray generator.

RESULTS

Preparation of water-soluble DR

Since crystallization of water-soluble proteins has generally been much more successful than crystallization of membrane-bound or detergent-soluble proteins, the crystallization of DR was approached by first preparing a water-soluble form. The preparation of a water-soluble form of DR that consists of the intact extracellular portion of the protein entails removing the transmembrane region and the cytoplasmic tail from each chain of the heterodimer (Korman *et al.*, 1985). It was shown previously that a controlled digestion of native DR, DQ and DP with papain would produce fragments that migrated like water-soluble proteins on polyacrylamide gels in the absence of SDS (Gorga *et al.*, 1987). Both chains of each class II molecule were cleaved, although the sizes of the resulting fragments varied from approximately 29,000 to 34,000 daltons for the alpha chains and from approximately 24,000 to 27,000 daltons for the beta chains. In order to further investigate the properties of the water-soluble DR, the migration of papain-digested DR1 ($DR1_{pap}$) on a gel filtration column in the absence and presence of detergent was compared with the migration of detergent-soluble DR1 and water-soluble and detergent-soluble HLA-A2 (fig. 1). Immunoaffinity-purified detergent-soluble DR1 (peak I, fig. 1A) migrated as a large molecule in the presence of 1 % deoxycholate. Because of the anomalous migration of many proteins in the presence of detergent (compare, for example, the migrations of BSA (MW 68,000) and IgG (MW 150,000) in the presence and absence of detergent), no attempt was made to quantitate the molecular weight. The detergent-soluble class I antigen HLA-A2 (peak II, fig. 1A) migrated as a smaller molecule than detergent-soluble DR1, presumably because it binds less detergent, since it has only one transmembrane region. The papain-solubilized forms of both DR1 and HLA-A2 ($A2_{pap}$) migrated as much smaller molecules, with the $DR1_{pap}$ peak (peak III, fig. 1A) eluting slightly before the $A2_{pap}$ peak (peak IV, fig. 1A). In the absence of deoxycholate, detergent-soluble DR1 and HLA-A2 did not migrate into the column, whereas $DR1_{pap}$ (peak III, fig. 2B) and $A2_{pap}$ (peak IV, fig. 2B) migrated at nearly the same position, again with $DR1_{pap}$ eluting slightly before (larger than) $A2_{pap}$.

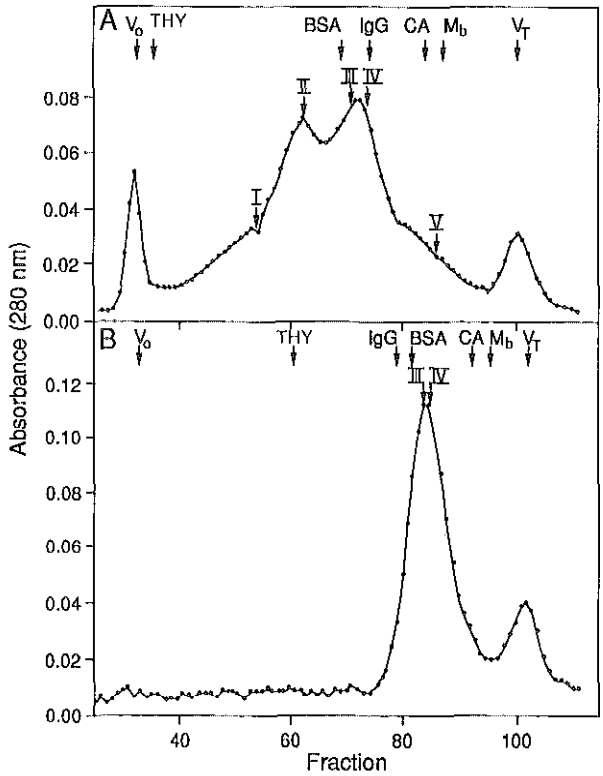

Fig. 1. Gel filtration of detergent- and water-soluble forms of DR1 and HLA-A2 on BioGel A5m.

Identical samples containing 500 μg each of detergent-soluble and papain-digested DR1 and detergent-soluble and papain-digested HLA-A2 were loaded in the presence (A) and absence (B) of 1 % (wt/vol) deoxycholate. Arrows mark peaks identified by SDS-PAGE as detergent-soluble DR1 (I), detergent-soluble HLA-A2 (II), papain-digested DR1 (III), papain-digested HLA-A2 (IV) and free β_2-microglobulin (V). In separate runs, a mixture of molecular weight standards was passed through the column in the presence or absence of 1 % deoxycholate. The migration of these standards is also marked with arrows: V_o, blue dextran; THY, thyroglobulin; IgG, immunoglobulin G; BSA, bovine serum albumin; CA, carbonic anhydrase; Mb, myoglobin; V_T, sodium p-nitrophenolate.

Large-scale papain digestion and purification for crystallization

For large-scale preparation of water-soluble DR, the papain digestion was carefully scaled up in order to keep the protein concentrations and ratio of papain to DR constant. For removal of papain, peptide fragments and detergent from the papain-digested DR1, the sample was loaded onto a Sephacryl S-200 column immediately after the digestion was completed. Often, a peak in the void volume containing residual Nonidet P-40, traces of papain and incompletely digested DR1 (peak I, fig. 2A) preceded the peak containing $DR1_{pap}$ (peak II, fig. 2A), and was not completely separated from it. Therefore, the fractions containing the $DR1_{pap}$ were combined and passed through a second gel filtration column (Sephadex G-150) in order to remove any remaining contaminating material. The fractions con-

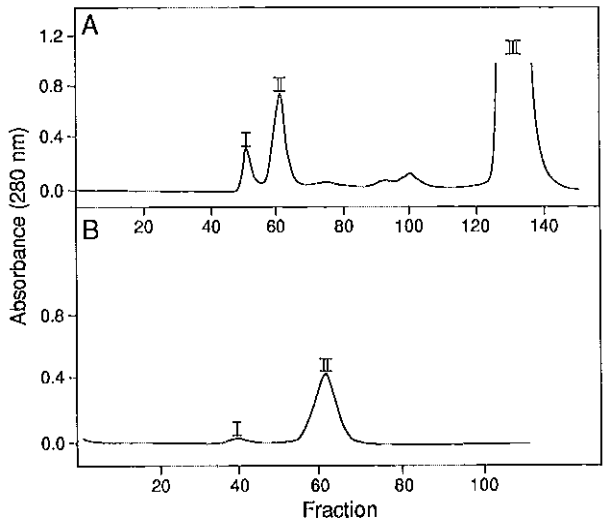

Fig. 2. Purification of papain-solubilized DR1 by gel filtration on (A) Sephacryl-S200 and (B) Sephadex-G150.

Immunoaffinity-purified DR1 (45 mg) was digested with 5.6 mg of papain for 60 min at 37° as described in "Materials and Methods". The digestion was stopped by the addition of 9 ml of 0.2 M iodoacetamide. The reaction mixture was loaded onto a 5.0×90 cm Sephacryl S-200 HR column at a flow rate of 100 ml/h, and 12.5 ml fractions were collected. Fractions 57-66 (peak II), identified by SDS-PAGE as containing papain-digested DR1, were combined, concentrated to approximately 5 ml and loaded onto a 2.6×70 cm Sephadex-G150 column at a flow rate of 5 ml/h. Fractions of 3.75 ml were collected.

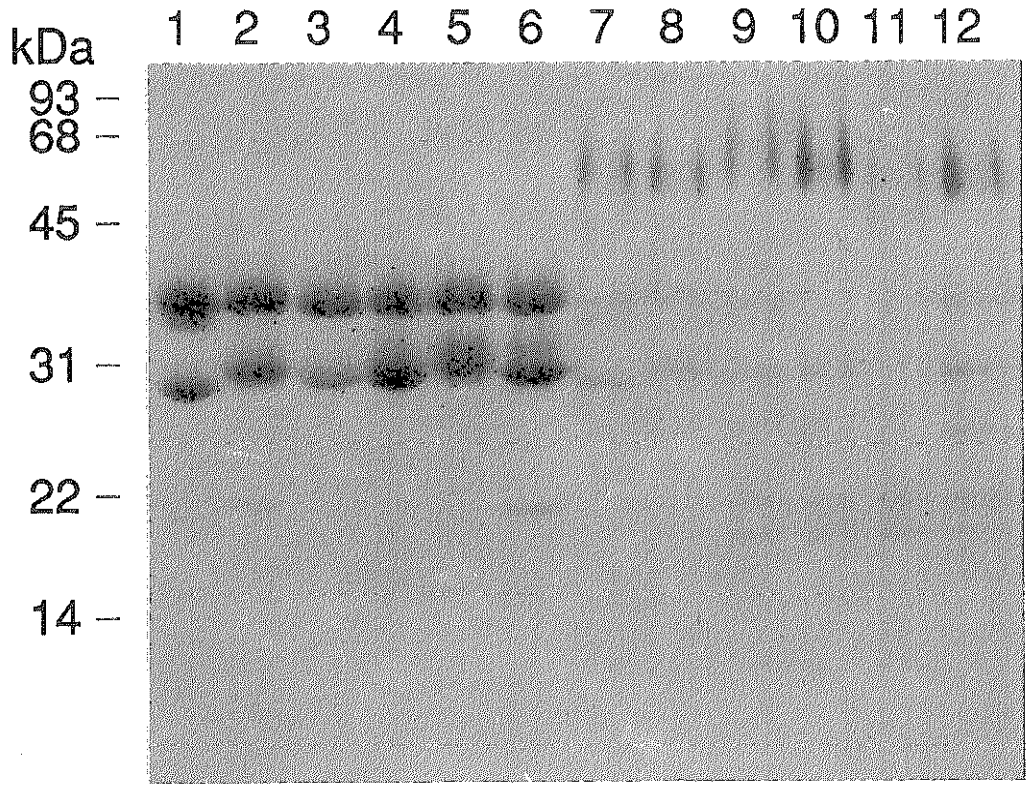

Fig. 3. SDS-PAGE analysis of papain-digested DR1, DR2, DR3, DR4, DR7, DR8 boiled (lanes 1-6, respectively) or not boiled (lanes 7-12, respectively) in Laemmli sample buffer.

For each sample, 5 μg of protein was run on a 12 % polyacrylamide gel. The gel was stained with Coomassie blue.

taining $DR1_{pap}$ (peak II, fig. 2B) were combined and concentrated by vacuum dialysis to approximately 10 mg/ml. The yield of $DR1_{pap}$ was 40-50 % of the amount of immunoaffinity-purified DR taken for the papain digestion.

Preparation of water-soluble forms of other DR alleles

Papain-solubilized DR2, DR3, DR4, DR7, and DR8 were prepared in the same manner from

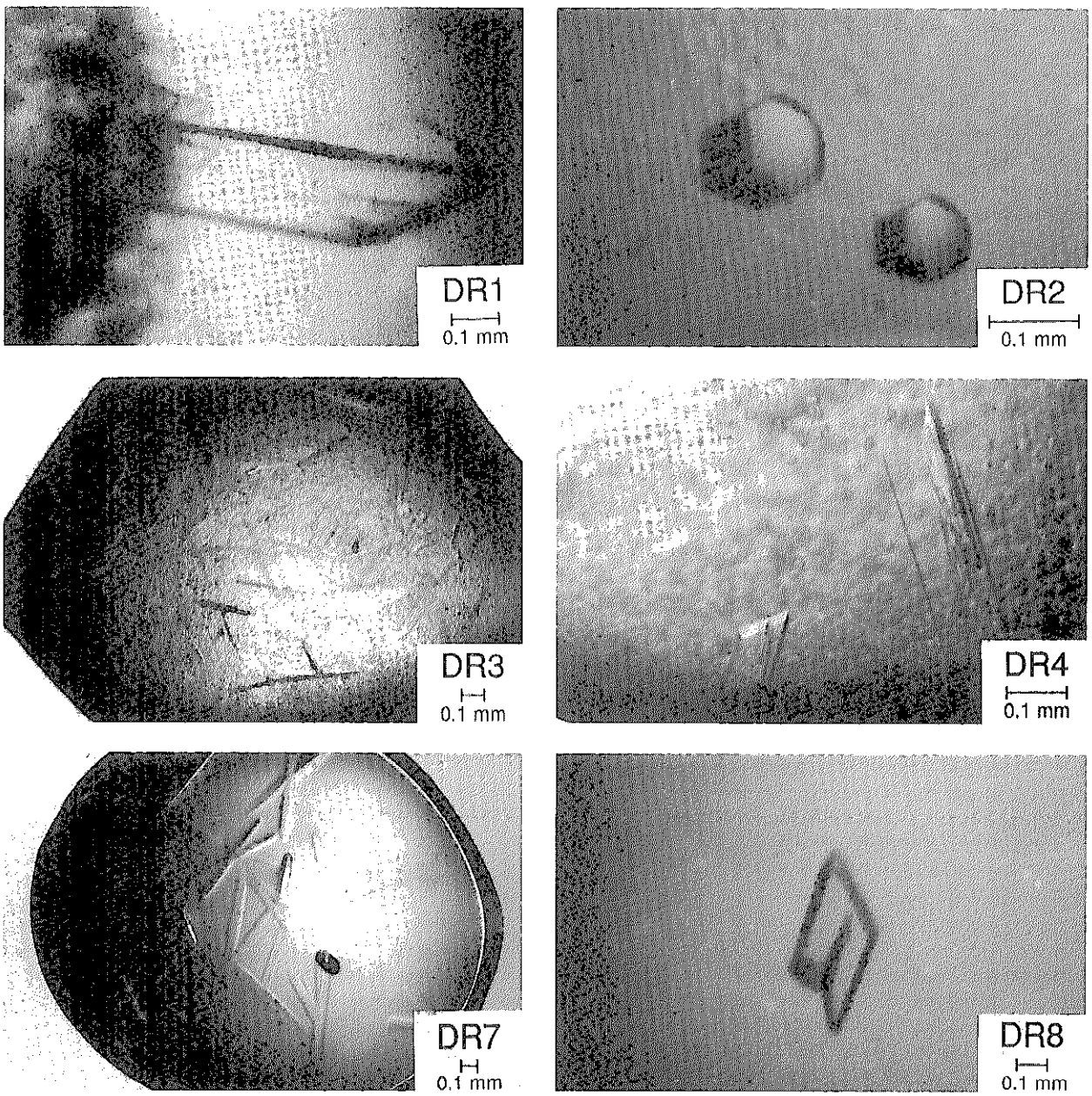

Fig. 4. Crystals of DR1 (grown from 28 % PEG 8000/100 mM glycine/0.01 % NaN_3, pH 6), DR2 (grown from 13 % PEG 3350/50 mM $MgCl_2$/100 mM acetate, pH 4.6), DR3 (grown from 16.8 % PEG 8000/56 mM $MgCl_2$/56 mM ADA, pH 4.7), DR4 (grown from 15 % PEG 8000/50 mM $MgCl_2$/67 mM ADA, pH 4.7), DR7 (grown from 17 % PEG 8000/100 mM NH_4SO_4/100 mM citrate, pH 6.1), DR8 (grown from 16 % PEG 8000/50 mM $MgCl_2$/67 mM ADA, pH 4.7).

The bar in each represents approximately 0.1 mm.

Table I. Comparison of crystals of DR subtypes.

DR subtype	Well condition	Maximum diffraction (Å)	Unit cell dimensions (Å)			Space group
DR1	100 mM glycine 20-28 % PEG 8000 0.01 % NaN_3 pH 3.7-6	3.5	96	112	209	$C222_1$ (*)
DR2	70 mM $MgCl_2$ 70 mM ADA 21 % PEG 8000 pH 4.7	(11)	97	112	211	$C222_1$
DR3	112 mM $MgCl_2$ 56 mM acetate 16.2 % PEG 3350 pH 4.9	3.7	97	111	211	(prob. $C222_1$)
DR4	96 mM $MgCl_2$ 48 mM acetate 14.4 % PEG 3350 pH 4.9	3.7	97	112	213	$C222_1$
DR7	92 mM $MgCl_2$ 46 mM acetate 13.8 % PEG 3350 pH 4.9	8	121	121	78.5	P6x
DR8	100 mM $MgCl_2$ 67 mM ADA 15 % PEG 8000 pH 4.7	8	96	112	210	(prob. $C222_1$)

Crystals of purified, papain-solubilized HLA-DR antigens were obtained as described in "Materials and Methods".
(*) Systematic absences corresponding to screw axes have been seen to at least 13 Å.

immunoaffinity-purified DR from the cell lines MST, WT20, Priess, Mann, and 23.1, respectively. The digested alpha and beta chains showed variability in their mobility on SDS-PAGE (fig. 3, lanes 1-6). Each of these preparations remained primarily in the form of alpha-beta complex in the absence of boiling in SDS (fig. 3, lanes 7-12).

Crystals of DR alleles

Crystals were obtained for each of the purified, papain-solubilized DR alleles (fig. 4). Two different orthorhombic crystal forms were obtained for $DR1_{pap}$. One of these diffracts as far as 3.5Å. Crystals of DR2, DR3, DR4 and DR8 have the same unit cell dimensions as the DR1 crystals, and crystals of DR3 and DR4 have the same diffracting power as the DR1 crystals (table I). Large hexagonal crystals that diffract to only about 8Å have been obtained for DR7. Crystals of similar hexagonal form have also been observed for most of the other DR subsets.

DISCUSSION

Water-soluble forms which appear to be intact extracellular fragments have been obtained for each of six different HLA-DR alleles. Crystals of each of these fragments were grown. Some of these crystals diffract to at least 3.5Å. It is perhaps noteworthy that with the exception of DR7, for which only hexagonal crystals have been obtained, all the DR alleles have crystallized in similar crystal forms. As far as we have determined from a limited number of crystals, the space groups and unit cell dimensions are the same for the different DR alleles. Thus, in spite of slight apparent differences in the fragments obtained by papain digestion, the DR molecules are likely to be very similar. Data collection and heavy atom derivative searches are proceeding.

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