Crystallization of HLA-DR4 fused to an immunodominant collagen II peptide implicated in rheumatoid arthritis

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Major histocompatibility complex (MHC) class II molecules are key players in antigen-specific $CD4^+$ T cell stimulation, despite their lack of discrimination between "self" and foreign antigens. The susceptibility of many individuals to autoimmune diseases is directly attributed to this lack of specificity, as well as to the expression of subclasses of MHC class II molecules. Increased susceptibility to the autoimmune disease rheumatoid arthritis (RA) has been attributed to the expression of the MHC class II alleles HLA-DR1 and HLA-DR4. To define the structural requirements of the HLA-DR4 protein in the autoimmune response of RA, we have crystallized HLA-DR4 with the immunodominant peptide from human collagen II, covalently linked to the *N*-terminus of the β-chain [HLA-DR4/hCII(257-273)]. Crystallization time, crystal size, and reproducibility were greatly improved by macroseeding into microdialysis buttons.

Keywords: HLA-DR4, collagen II, rheumatoid arthritis, microdialysis, protein crystallization

1. Introduction

Antigen-specific $CD4^+$ T-cell stimulation is directly mediated by the action of the polymorphic major histocompatibility complex (MHC) class II family of cell surface molecules. The allelic polymorphisms found within class II molecules directly affect the peptide binding affinities of MHC class II for their peptide ligands, which subsequently affects the immunogenicities of the peptides that are presented. MHC class II molecules are unique in that they fail to discriminate between peptides generated from normal host proteins ("self" antigens) and those generated from foreign proteins (Rosloniec *et al.*, 1990, Benichou *et al.*, 1990). As would be expected, many of the peptides bound by MHC class II molecules are generated from "self" proteins, which would put a majority of the population at risk to developing autoimmune diseases. However, such pervasive autoimmunity is held in check by tight regulation of T cell development and stimulation within the immune system (Wong *et al.*, 2001). This strict regulation is, however, not absolute, and a significant portion of the population does develop autoimmune diseases. The exact etiology of most these diseases is unknown, but MHC class II molecules have been implicated as culprits. For example, the increased risk of developing the autoimmune disease rheumatoid arthritis (RA) has been attributed to the expression of the MHC class II alleles HLA-DR1 and HLA-DR4, specifically the DRB1*0101, DRB1*0401, DRB1*0404, or DRB1*0405 alleles (Statsny *et al.*, 1988). However, it still remains to be determined how the presence of these particular molecules predisposes an individual to developing RA. Numerous hypotheses aimed at determining the functional role of these class II molecules in RA have been proposed (Winchester & Dwyer, 1991, Albani *et al.*, 1992, Zanelli *et al.*, 1995), but identification of the causative agents of the disease remain obscure.

A wide variety of proteins are involved in the stimulation of pathogenic T cells in RA, including proteins from cytomegalovirus (Fujinami *et al.*, 1988), Epstein-Barr virus (Roudier *et al.*, 1988), and several proteins found in the joints, including gp39 (Cope *et al.*, 1999), proteoglycan (Banerjee & Poole, 1992), and type II collagen (Stuart *et al.*, 1988). In particular, many studies have focused on the specific role of type II collagen (CII) in RA, due in part to its presence as a major component of articular cartilage, and the demonstrated cellular immunity to CII in RA patients (Watson *et al.*, 1994, Kim *et al.*, 1999; 2000).

Through the use of transgenic mice, it has been determined that HLA-DR1 and HLA-DR4 bind antigenic peptides from human CII (hCII) and mediate the susceptibility to RA in the mouse (Rosloniec *et al.*, 1997; 1998). Both of these molecules bind to and are involved in antigenic presentation of the same hCII immunodominant peptide, hCII(257-273), and both use Phe263 and Lys264 of the peptide as binding anchors (Rosloniec *et al.*, 2002). The use of only these two adjacent residues as binding anchors is quite different from all other HLA-DR1- and HLA-DR4-peptide interactions, as are the low affinities of both molecules for hCII. The structure of a CII peptide bound by HLA-DR4 (DRB1*0401) has been determined (Dessen *et al.*, 1997). However, the peptide used in these analyses was from a procollagen portion of the CII molecule that is not part of mature cartilaginous tissue. Using this crystal structure as a template, the authors modelled hCII(261-273) into the HLA-DR4 binding cleft. Several hCII residues predicted to interact with DR4 based upon this model were later confirmed (Andersson *et al.*, 1998). To determine the structural specifics in the binding of hCII to HLA-DR4 we crystallized HLA-DR4 with the immunodominant peptide hCII(257-273) covalently linked to the *N*-terminus of the β-chain [HLA-DR4/hCII(257-273)].

2. Materials and methods

2.1. Crystallization of HLA-DR4 linked to the immunodominant human CII peptide (257-273).

Purified and biologically active samples of recombinant HLA-DR4/hCII(257-273) were the generous gift of Dr. Ed Rosloniec. The sequence of the β-chain of HLA-DR4 was modified by PCR insertion mutagenesis to incorporate amino acids 257-273 of hCII into the expressed β-chain peptide using the method of Crawford *et al*. (1998). The protein was concentrated to 10 mg/ml for crystallization trials. Initial crystals were obtained at room temperature from 18% polyethylene glycol of molecular weight 3000 (PEG-3000; Fluka Chemical) and 0.1 M Tris at pH 8.0 (Sigma Chemical Co.). Macroseeding into microdialysis buttons was undertaken to improve crystal size. Following microdialysis for three days, we removed seed crystals and washed them sequentially in 20% w/v PEG-3000, 18% w/v PEG-3000, and 16% w/v PEG-3000, and incubated in 16% w/v PEG-3000 including 2 mg/ml HLA-DR4/hCII(257-273) for five minutes. PEG of molecular weights 3000 and 4000 were used as precipitants in parallel trials and 0.1 M Tris at pH 8.0 was used in all wash solutions. The washed crystals were placed in 5 µl dialysis buttons containing 18% PEG of either molecular weight 3000 or 4000, 0.1 M Tris at pH 8.0, and 10 mg/ml HLA-DR4/hCII(257-273). The microdialysis button were placed in 5 ml of the same crystallization solution and incubated at room temperature.

2.2. X-ray diffraction of HLA-DR4/hCII(257-273) crystals

Crystals were screened for X-ray diffraction using a DIP2030 area detector system (MacScience) mounted on a Nonius FR591 X-ray generator operating at 45 kV and 90 mA equipped with focusing

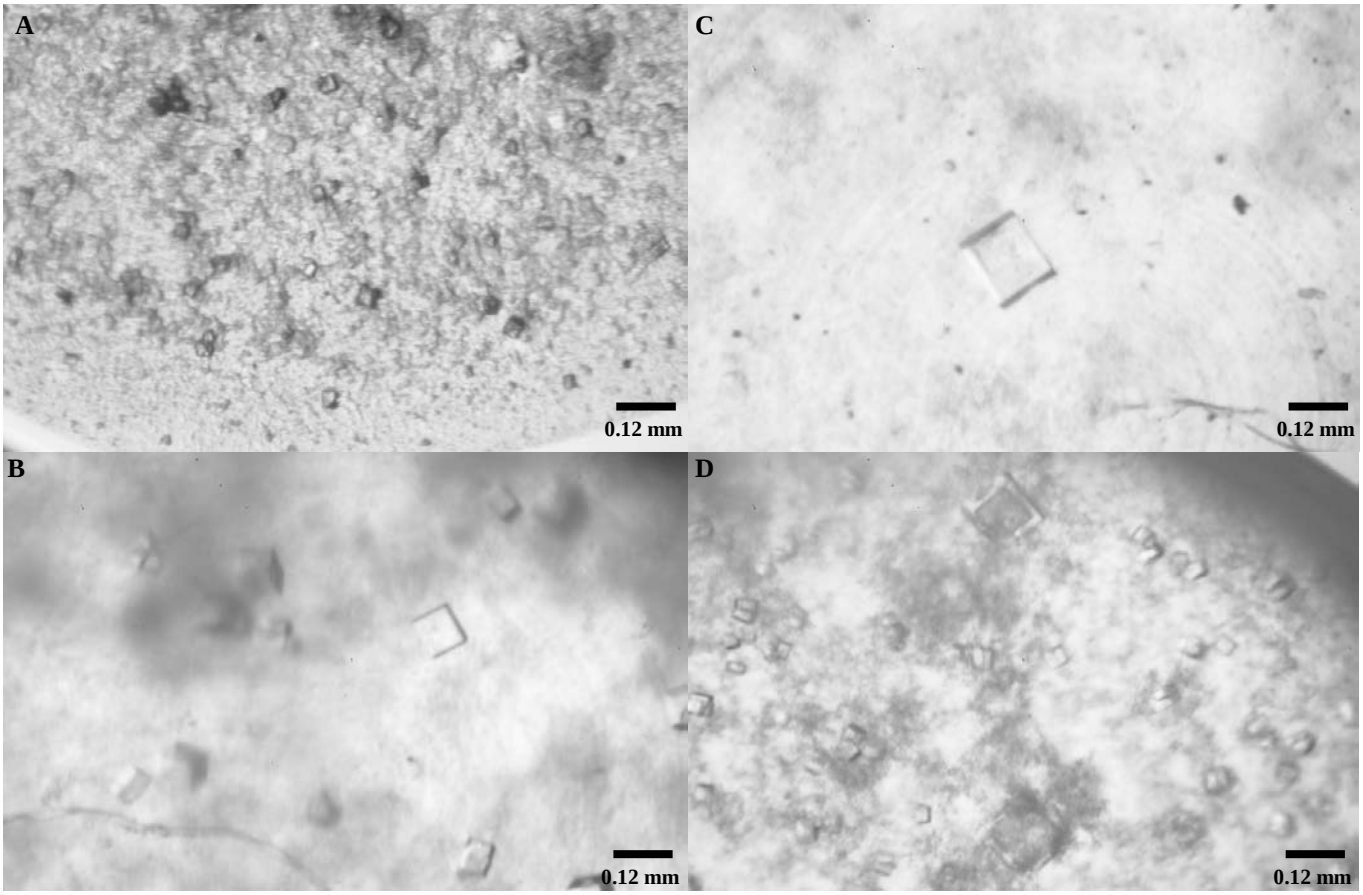

Figure 1 Photomicrographs of HLA-DR4/hCII(257-273) crystals. Crystals obtained from initial crystallization screens (**A**) using PEG as the precipitating agent and 0.1 M Tris at pH 8.0, stained with IZIT. Microdialysis resulted in the crystals seen in **B**, obtained using similar conditions. Using these crystals as seeds, the crystals seen in **C** and **D** were obtained from 0.1M Tris at pH 8.0 and PEG-3000 or PEG-4000, respectively. All photographs were taken at the same magnification.

mirrors (MacScience). Crystals were soaked in 0.1 M Tris pH 8.0, 16% PEG-3000, and 30% glycerol prior to cryo-freezing for X-ray diffraction analysis.

3. Results and discussion

Vapour diffusion was used to obtain crystals of HLA-DR4/hCII(257-273). These crystals grew over a period of 2 months to a final size of 0.02 mm in all three dimensions (Figure 1A). To confirm that we had indeed crystallized HLA-DR4/cHII(257-273), we performed an SDS page gel analysis of the crystals and stained the crystals with the protein dye IZIT (Hampton Research; Figure 1A). Microdialysis methods were used to increase the size of the crystals. This approach not only increased the crystal size to 0.08 mm in all three dimensions (Figure 1B) but, more importantly, allowed crystal growth within only 24 hours. These crystals diffracted to 6.5 Å Bragg spacing (data not shown). To obtain larger crystals with stronger diffraction to higher resolution, seed crystals were placed into microdialysis buttons, which generated larger crystals (Figures 1C and 1D). Using PEG-3000 as the precipitating agent in the wash and in the dialysis solutions crystals of up to 0.12 mm in each dimension were obtained (Figure 1C). By contrast, in crystallization experiments utilizing PEG-4000 as the precipitant, dense protein precipitation on the surface of the seed crystals was observed during the initial 24 hours of dialysis (Figure 1D). This was followed by visible increases in crystal size during the ensuing 24 - 36 hours, resulting in a final average size of 0.12 mm in each dimension. The dense precipitation initially observed using PEG-4000 could be due to the increased degree of polymerization that drives faster protein aggregation, as this precipitation was not observed in samples using the smaller PEG-3000. Unfortunately, despite their improved size, crystals grown from the seeds did not display significant improved diffraction. Poor diffraction was observed of crystals of HLA-DR1 has been previously reported (Dessen *et al*., 1997) and high quality crystals required co-crystallization with staphylococcal enterotoxin B (SEB). We are currently co-crystallizing HLA-DR4/hCII(257-273) with SEB. However, the described method of macroseeding into dialysis buttons reported here is novel and could potentially be of general use for many proteins by both decreasing crystal growth time and increasing crystal size.

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