IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ZYMOGENETICS, INC., a Washington Corporation,	)	
Plaintiff,	)	Civil Action No: 06-500-SLR-MPT
v.	)	
BRISTOL-MYERS SQUIBB CO., a Delaware Corporation, and DOES 1 through 100,	)	**REDACTED PUBLIC VERSION**
Defendants.	)	

DECLARATION OF DAVID M. KELLY IN SUPPORT OF BRISTOL-MYERS SQUIBB COMPANY'S ANSWERING BRIEF OPPOSING ZYMOGENETICS INC.'S MOTION FOR SUMMARY JUDGMENT OF INFRINGEMENT

I, David M. Kelly, hereby declare the following:

1. I am an associate with the law firm of Fitzpatrick, Cella, Harper & Scinto, attorneys for Defendant Bristol-Myers Squibb Company in this action. I submit this Declaration in support of Bristol's Answering Brief Opposing ZymoGenetics, Inc.'s Motion for Summary Judgment of Infringement of the Patents-In-Suit.

2. Attached hereto as Infringement Answering Brief ("I.A.B.") Exhibit 1 is a true and correct copy of excerpts of the Transcript of the August 21, 2007 Deposition of Andrzej Sledziewski, Ph.D., pp. 45-48, 89-92.

3. Attached hereto as I.A.B. Exhibit 2 is a true and correct copy of excerpts of the Transcript of the April 29, 2008 Deposition of Nicholas R.J. Gascoigne, Ph.D., pp. 162-167, 172.

4. Attached hereto as I.A.B. Exhibit 3 is a true and correct copy of excerpts of the Transcript of the June 27, 2008 Markman Hearing before the Honorable Sue L. Robinson, pp. 61-62.

5. Attached hereto as I.A.B. Exhibit 4 is a true and correct copy of excerpts of the Transcript of the May 6, 2008 Deposition of David A. Ostrov, Ph.D., pp. 29-32.

6. Attached hereto as I.A.B. Exhibit 5 is a true and correct copy of excerpts of the Transcript of the September 15, 2007 Deposition of Peter Linsley, Ph.D., pp. 37-40.

7. Attached hereto as I.A.B. Exhibit 6 is a true and correct copy of excerpts of the Transcript of the May 20, 2008 Deposition of Jeffrey V. Ravetch, M.D., Ph.D., pp. 165-168.

8. Attached hereto as I.A.B. Exhibit 7 is a true and correct copy of Zhang, X. et al., *Expression, Refolding, Purification, Molecular Characterization, Crystallization, and Preliminary X-ray Analysis of the Receptor Binding Domain of Human B7-2*, Protein Expression and Purification, 25:105-113 (2002).

I declare under penalty of perjury under the laws of the United States of America that the forgoing is true and correct.

Executed this 18th day of August, 2008, in New York, New York.

David M. Kelly

Exhibit 1 Redacted In Its Entirety

Exhibit 2 Redacted In Its Entirety

EXHIBIT 3

- TRANSCRIPT UNDER SEAL -

IN THE UNITED STATES DISTRICT COURT

IN AND FOR THE DISTRICT OF DELAWARE

- - -

ZYMOGENETICS, INC., a Washington Corporation,	:	CIVIL ACTION
Plaintiff,	:	
vs.	:	
BRISTOL-MYERS SQUIBB CO., a Delaware Corporation, and DOES 1 through 100,	:	
Defendants.	:	NO. 06-500 (SLR)

- - -

Wilmington, Delaware
Friday, June 27, 2008
9:00 o'clock, a.m.

- - -

BEFORE: HONORABLE SUE L. ROBINSON, U.S.D.C.J.

- - -

APPEARANCES:

POTTER, ANDERSON & CORROON
BY: PHILIP A. ROVNER, ESQ.

-and-

construction is, the common sense we think of a construction, based on prosecution specification.

Now, we did go through that with their experts again, to see if they agreed with that interpretation. Again, this is Dr. Gascoigne, two of ZymoGenetics' experts.

As a scientist reading the patent, is it your understanding that but for the immunoglobulin component of the claimed fusion protein, the non-immunoglobulin component of the fusion protein wouldn't dimerize?

He agreed.

We also asked their inventor, Dr. Kindsvogel, what's causing the dimerization here? What's causing dimerization?

He said, It's being driven by the immunoglobulin portion.

That's the solution to their problem, that their dimerizing protein is putting together something that wouldn't go together by itself. And that's why we asked them the construction that we asked for.

THE COURT: All right. Anything else on that particular --

MR. ANDRE: Your Honor, just very briefly, your Honor. I mean, I'm not going to, obviously, make the -- the obvious argument, that's all extrinsic evidence and not related to the patent. Every time you see something

related to the patent, it's in a preferred embodiment whether it is driving or not.

I want to talk about the second element here, because I think the two about driving is very clear and not in the extrinsic evidence. Putting the term dimerizing protein in context of the claim is very important. Your Honor stated it was passive. With all due respect, I don't think it is necessarily passive.

THE COURT: More passive.

MR. ANDRE: The definition. The definition is a polypeptide chain having affinity for a second polypeptide chain, such that the two chains associated -- they associate under physiological conditions.

I think if a change associates in the biologic context, that's not passive. These chains associate each other. Association and polypeptide chains means they are coming together through some type of interaction. They associate under physiological conditions to form a dimer.

So with all due respect, I don't think they are necessarily passive to two skilled in the art. I think it does describe what's going on.

More importantly, how that term is read in context of the claim, you have two portion of the fusion, molecule, joined to a dimerizing protein, heterologous to said ligand-binding or heterologous to non-Ig.

Exhibit 4 Redacted In Its Entirety

Exhibit 5 Redacted In Its Entirety

Exhibit 6 Redacted In Its Entirety

EXHIBIT 7

Protein Expression and Purification 25, 105–113 (2002)
doi:10.1006/prep.2002.1616

Expression, Refolding, Purification, Molecular Characterization, Crystallization, and Preliminary X-ray Analysis of the Receptor Binding Domain of Human B7-2

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Received November 26, 2001, and in revised form January 15, 2002

The cell-mediated immune response involves a series of specific molecular interactions between cell surface molecules on T cells and antigen-presenting cells. Of particular importance for the regulation of T cell activity is the interaction of the B7 isoforms, B7-1 and B7-2, with the T cell surface costimulatory receptors, CD28 and CTLA-4. The binding of CD28 by B7-1/B7-2 results in an enhancement of T cell responses initiated by the interaction between a clonotypic T cell receptor and its specific, antigenic MHC–peptide complex, whereas the subsequent engagement of CTLA-4 by B7-1/B7-2 leads to a down-regulation of the response. Here we report the expression, refolding, purification, characterization, and crystallization of the receptor-binding domain of human B7-2. The receptor-binding domain of human B7-2 was overexpressed in *Escherichia coli* as inclusion bodies, solubilized in 6 M guanidine–hydrochloride, and then refolded *in vitro* by rapid dilution into a renaturing buffer. Refolded B7-2 was subsequently purified to homogeneity by anion-exchange chromatography. Gel-filtration chromatography and native PAGE analysis showed that the receptor-binding domain of B7-2 is exclusively monomeric in solution. Purified B7-2 binds tightly to bacterially expressed monomeric and disulfide-linked homodimeric human CTLA-4 as shown by gel-filtration chromatography and native PAGE. This suggests that glycosylation is not important for the proper folding of the receptor-binding domain of B7-2 nor for its binding to CTLA-4. In addition, these results suggest that refolded B7-2 is biologically active and may be a useful therapeutic and experimental reagent for regulating T cell activity. Refolded and purified B7-2 was crystallized by the hanging-drop vapor diffusion method, allowing for the initiation of an X-ray crystallographic study. © 2002 Elsevier Science (USA)

T cells require two signals resulting from distinct receptor recognition events for efficient activation (1). The first signal conveys information to the T cell about the specificity of the response and results from atomic interactions between the T cell receptors (TCRs) on the surfaces of T cells and peptide fragments that derive from either self or foreign sources, bound to germ-line-encoded major histocompatibility complex (MHC) molecules on the surfaces of antigen-presenting cells (APCs). This interaction alone is not sufficient for T cell activation and in the absence of a second, simultaneous signal would result in either apoptotic death or prolonged unresponsiveness (anergy) of the responding T cell (2). The required second signal results from the engagement of invariant T cell surface costimulatory receptors by their respective ligands on the surfaces of APCs. The most well-characterized T cell costimulatory receptor is CD28, which binds both B7-1 and B7-2 ligands on APCs (3–6). Together with signaling through the TCR, the engagement of CD28 by the B7 ligands leads to optimal T cell activation, characterized by vigorous T cell proliferation and cytokine secretion (5, 7). In contrast, the subsequent engagement of the B7 ligands with CTLA-4, a CD28 homologue also expressed on the surfaces of T cells, leads to attenuation of the response (8, 9). While

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both CD28 and CTLA-4 are predominantly disulfide-linked homodimers on the surfaces of T cells (9–12), a small fraction of both receptors has been observed to exist in monomeric form (9, 13).

B7-1 and B7-2 are homologous (~26% identity), glycosylated type I transmembrane proteins consisting of a membrane-distal V-type immunoglobulin (Ig) domain and a membrane-proximal C-type Ig domain (6). Despite their similar structures and binding to the same receptors (14–16), B7-1 and B7-2 do exhibit some distinct immunoregulatory functions. B7-2 and CD28 are constitutively expressed on APCs and T cells, respectively, and their interaction is thought to provide the predominant, initiating costimulatory signal to naïve T cells within draining lymphoid tissues (1, 6, 17). In contrast, B7-1 is expressed at low levels on resting APCs and it is up-regulated only after immune activation (3, 4). B7-1 is therefore thought to be the more important ligand at later phases of the immune response for the purposes of sustaining T cell costimulation at effector sites (17). Twenty-four to forty-eight hours postactivation, T cells induce cell surface levels of CTLA-4, which both competes with CD28 for binding to B7 and directs the assembly of inhibitory signaling complexes to terminate the immune response (8, 13, 18). Biochemical differences between B7-1 and B7-2 have been observed in that B7-1 has ~10-fold higher affinity than B7-2 for their receptors, CD28 and CTLA-4 (19, 20). Another important difference observed between the two B7 isoforms is that while both the V-type and the C-type Ig domains of B7-1 are required for optimal binding to its receptors (21), the V-type Ig domain of B7-2 exhibits the same binding affinity for both CD28 and CTLA-4 as the full-length B7-2 (22, 23). Biochemical and crystallographic data have revealed that the extracellular region of human B7-1 is dimeric both in solution and in crystalline form (14, 16). Even though structural analysis demonstrates that the residues responsible for both dimerization and receptor binding are exclusively located within the V-type Ig domain, close inspection of the B7-1 structure reveals a significant number of contacts between the V-type and the C-type Ig domains, which may explain why both domains are required for optimal receptor binding.

In an effort to understand the molecular basis for the observed differences between B7-1 and B7-2, we established a bacterial expression system for the production of recombinant human B7-2 for biochemical and X-ray crystallographic studies. In addition, as soluble human B7-2 has been shown to be a functional costimulatory molecule for human T cells (24), development of an efficient biosynthetic route to produce large quantities of this molecule should be useful in both experimental and clinical settings. There is only one previous report of the production of an active B7 molecule from a bacterial expression system which, in our hands, produced microgram levels of active protein per liter of bacterial culture (25). Here we describe a method for the production of the receptor-binding (V-type Ig) domain of human B7-2 (hB7-2V) with yields approaching 35 mg of functional protein per liter of bacterial culture. In addition, we describe the crystallization and preliminary X-ray analysis of this important costimulatory ligand.

MATERIALS AND METHODS

Materials and chemicals. Synthetic DNA oligonucleotides were ordered from Invitrogen Life Technologies, Inc. Restriction endonucleases were from New England Biolabs or Boehringer Mannheim. The pPCR-Script Amp SK(+) vector and *Pfu* DNA polymerase were purchased from Stratagene. The pET3a vector and *Escherichia coli* strains were from Novagen. Plasmid DNA was extracted using a spin miniprep kit from Qiagen. All chromatography columns and equipment used were purchased from Amersham Pharmacia Biotech. Protein was concentrated using Amicon concentrators and ultrafiltration membranes. Crystallization trials were performed using reagents from Hampton Research. Precast native gels were obtained from Bio-Rad. All other chemicals were of analytical grade.

Cloning of hB7-2V and monomeric and disulfide-linked dimeric human CTLA-4. The cDNA for the first 109 amino acids (V-type Ig domain) of the extracellular region of B7-2 was amplified by PCR from a template obtained from ATCC containing the complete coding sequence of this protein (ATCC Item 99597, DNA Sequence Accession No. U04343). The forward oligonucleotide, 5′GATCCATATGCTGAAGAATCAAGCTTATTTCAA3′, incorporated an *Nde*I restriction site (which also encodes the initiator methionine residue) at the 5′ end of the gene, and the reverse oligonucleotide, 5′GATCGGATCCTCAAGCAAGCACTGACAGTTCAG3′, incorporated a stop codon and a *Bam*HI restriction site at the 3′ end of the gene. The PCR product was ligated into the pPCR-Script Amp SK(+) vector and transformed into the *E. coli* strain DH5α. Positive colonies were selected and cultured for plasmid extraction. The nucleotide sequence was confirmed by DNA sequencing. The insert was digested with *Bam*HI and *Nde*I and ligated into the bacterial expression vector, pET3a, which had been predigested with the same restriction enzymes. Monomeric (residues 1–121 of the V-type Ig domain) and disulfide-linked homodimeric (residues 1–126, including Cys122, which forms the interchain disulfide) human CTLA-4 were cloned similar to hB7-2V (12). The forward oligonucleotide for both monomeric and dimeric CTLA-4 was 5′GATCCATATGAAAGCAATGCACGTGGCCC3′, while the reverse oligonucleotides for monomeric and dimeric CTLA-4 were 5′GATCGGATCCTCACGGTTCTGGATCAATTACATA3′ and

5′GATCGGATCCTCAGTCAGAATCTGGGCACGGT3′, respectively.

Expression, refolding, and purification of hB7-2V and monomeric and disulfide-linked dimeric human CTLA-4. The pET3a vector carrying the insert for hB7-2V and an initiator methionine was used to transform the competent *E. coli* strain BL21(DE3)plysS for recombinant protein expression. Transformed cells were grown at 37°C in Luria–Bertani (LB) medium supplemented with 50 μg/ml carbenicillin until OD_{600} reached 0.5–0.7, at which time the cultures were induced by the addition of 1 mM isopropylthiogalactosidase (IPTG). Incubation was then continued for 3 h under the same conditions. Cells were harvested by centrifugation at 5000*g* at 4°C for 20 min and the cell pellet was resuspended in lysis buffer (~5 ml lysis buffer per liter of bacterial culture) composed of 10 mM Tris–HCl, 150 mM NaCl, 25% (w/v) sucrose, 1 mM EDTA, and 10 mM dithiothreitol (DTT), pH 8.0. Cells were lysed by passing the mixture three times through a french pressure cell at 1000 psi. hB7-2V, expressed as inclusion bodies, was recovered by centrifugation at 10,000*g*, 4°C for 15 min. Inclusion bodies were washed three times with buffer containing 10 mM Tris–HCl, 100 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, and 10 mM DTT, pH 8.0, and then one final time with the same buffer excluding Triton X-100. Washed inclusion bodies were dissolved in buffer containing 10 mM NaAc, 6 M guanidine–hydrochloride (Gu-HCl), 5 mM EDTA, and 1 mM DTT, pH 4.6. The solubilized inclusion bodies were centrifuged at 15,000*g* and then passed through a 0.45-μm syringe filter. Protein concentration was determined by absorption at 280 nm based on the theoretically calculated extinction coefficient (16,500 M^{-1} cm^{-1}). The solubilized material was refolded by the fast dilution method described by Garborczi *et al.* (1996) with minor modifications (26). Sixteen milligrams of solubilized inclusion bodies were diluted into 16 ml of buffer composed of 10 mM NaAc, 6 M Gu-HCl, 5 mM EDTA, pH 4.6, and rapidly diluted over a few seconds into 1 liter of refolding buffer composed of 100 mM Tris–HCl, 0.4 M arginine–HCl, 1 mM EDTA, 5 mM cysteamine, 0.5 mM cystamine, pH 8.5, at 4°C with vigorous stirring. Five additional aliquots of hB7-2V (16 mg each) were added every 6–8 h. Refolding was monitored by gel-filtration chromatography using a Superdex G-75 column (30 × 1.0) at a flow rate of 0.75 ml/min with buffer composed of 10 mM Tris–HCl, 50 mM NaCl, pH 8.0. Refolded hB7-2V was concentrated and buffer exchanged to 10 mM Tris–HCl, 20 mM NaCl, pH 8.5, using a 350-ml Amicon concentration cell with a 3K molecular weight cutoff ultrafiltration membrane. The protein solution was centrifuged at 15,000*g*, passed through a 0.45-μm syringe filter, and loaded onto a prepacked 1-ml MonoQ column preequilibrated with 10 mM Tris–HCl, pH 8.0, at 1 ml/min using an AKTA Explorer chromatography system. A 0–500 mM NaCl linear gradient spanning 20 column volumes (20 ml) was used for protein elution. Typically, hB7-2V eluted at ~150 mM NaCl. The oligomeric state and purity of hB7-2V were determined by gel-filtration chromatography and SDS–PAGE, respectively. Protein was concentrated to about 10 mg/ml and buffer exchanged to 10 mM Tris–HCl, 20 mM NaCl, pH 8.0, using an Amicon concentrator with a 5K molecular weight cutoff membrane. hB7-2V was stable for months at −70°C.

Incorporation of selenomethionine into the recombinant protein would allow for the use of the multiwavelength anomalous dispersion (MAD) method to solve the crystallographic phase problem (27). In order to label the recombinant protein with selenomethionine, the competent bacterial strain B834, a methionine auxotroph, was transformed with the pET3a plasmid containing the cDNA for hB7-2V. M9 medium (M9 salt, 1% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mg/liter thiamine, 7.5 mg/liter $FeSO_4$, 5 mg/liter of both tryptophan and tyrosine, 50 mg/liter of selenomethionine and all other amino acids except cysteine) supplemented with 5% LB medium and 50 μg/ml carbenecillin was inoculated with a single B834 transformant colony for culture at 37°C overnight. Cells were harvested by centrifugation at 5000*g* and resuspended in M9 medium supplemented with 50 μg/ml carbenecillin (without supplementation of LB) and allowed to grow at 37°C until the absorbance of the culture at 600 nm reached 0.5–0.7. Recombinant protein production was then induced by the addition of 1 mM IPTG and further culture for 5–7 h under the same conditions. All remaining procedures were the same as those described for the native protein except for the addition of 1 mM DTT to all buffers to minimize air oxidation of selenomethionines (we did not include 1 mM DTT, however, during the refolding procedure).

Monomeric CTLA-4 was refolded using the same procedure as described for hB7-2V, while the refolding of the disulfide-linked homodimer of human CTLA-4 has been previously reported by Cox *et al.* (28). Monomeric and dimeric CTLA-4 were purified by a combination of anion-exchange and gel-filtration chromatography as described above. Purified proteins were concentrated to ~10 mg/ml and buffer was exchanged into 10 mM Tris–HCl, 20 mM NaCl, pH 8.0, using Centricon concentrators with 5K molecular weight cutoff membranes. Protein was stable at −70°C for several months.

Binding assays with human CTLA-4. hB7-2V and either monomeric or disulfide-linked dimeric CTLA-4 were mixed at 1:1, 1:4, and 4:1 molar ratios (based on monomer concentration) and incubated overnight at 4°C. Gel-filtration chromatography and native PAGE analysis were used to detect the formation of monomeric

and dimeric CTLA-4/B7-2 complexes. hB7-2V and monomeric and disulfide-linked CTLA-4 were run individually on both gel-filtration and native gels as standards. Gel-filtration chromatography was performed as described above for protein purification. Native gels were 10–20% gradient polyacrylamide with 0.75-mm thickness. Standard Tris/Glycine buffers without SDS were used as running buffers. Five to twenty microliters of protein mixtures was mixed with an equal volume of 2× loading buffer (100 mM Tris–HCl, 0.2% bromophenol blue, 20% glycerol, pH 6.8) and loaded onto gels without heating or reduction. Electrophoresis was performed at 70 V for 7–9 h at 4°C.

Crystallization and data collection. Initial crystallization trials were carried out by the method of hanging-drop vapor diffusion at room temperature and 4°C. Two microliters of protein solution of native hB7-2V at 3.75 mg/ml was mixed with 2 μl reservoir solution and equilibrated against 0.75 ml of reservoir solution. Twinned crystals were obtained under several conditions at room temperature that contained ammonium sulfate; however, exhaustive screening of these crystallization conditions failed to produce useful single crystals. Plate and needle-like crystals were obtained from PEG 4000, and after optimization, diffraction-quality crystals were obtained in 0.1 M NaAc, 0.1 M Tris–HCl, 16–26% PEG 4000, pH 8.5 at 4°C. Selenomethionine-substituted protein was crystallized under similar conditions in the presence of 1 mM DTT. Crystals were cryoprotected by adding 2 μl reservoir buffer containing 30% glycerol into a 2-μl crystal drop and flash freezing them at 100 K. A set of diffraction data on a single native crystal was collected at 2.3 Å resolution at beamline X-9B (NSLS) using an ASDC-CCD detector. The presence of selenomethionine in the derivative crystals was confirmed by X-ray fluorescence. A single selenomethionine-substituted protein crystal was used to collect a data set to 2.7 Å resolution, using the three wavelength MAD technique at wavelengths 0.98019, 0.98000, and 0.96110 Å. Diffraction data were processed with the HKL suite (29).

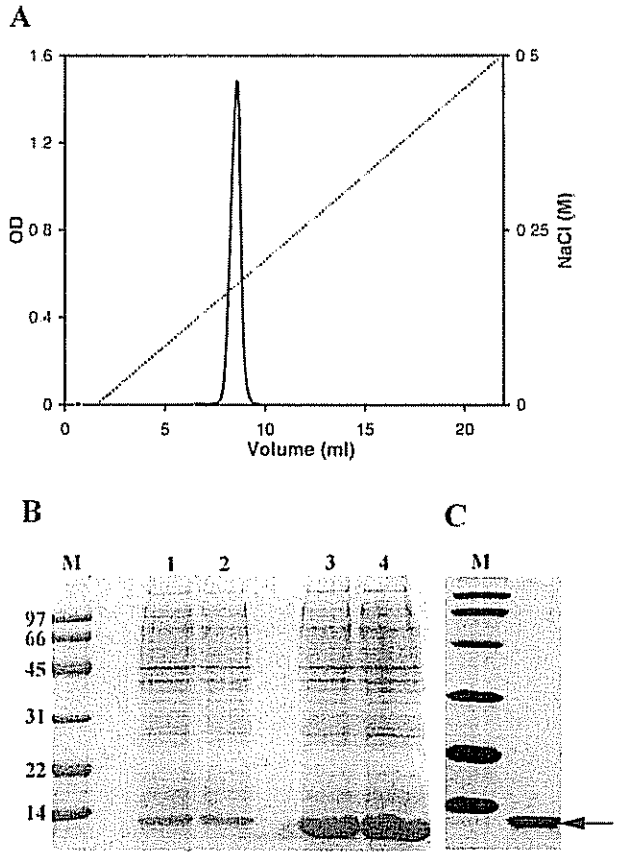

FIG. 1. Expression and purification of refolded hB7-2V. (A) Elution profile of refolded hB7-2V from MonoQ anion-exchange column chromatography. Fractions corresponding to the protein peak were pooled and subjected to further analysis. (B) Expression of hB7-2V analyzed by reducing SDS–PAGE. SDS–PAGE was performed with 15% polyacrylamide gels and stained with Coomassie blue R250. Lanes 1 and 2 correspond to lysates from *E. coli* before induction, while lanes 3 and 4 correspond to lysates from *E. coli* 3 h after induction with 1 mM IPTG. Note that hB7-2V was expressed at low levels even in the absence of IPTG induction. (C) Purified hB7-2V analyzed by SDS–PAGE. The arrow indicates the bands of hB7.2V (MW 12,834) in both (B) and (C). The single, intense band of hB7-2V in (C) indicates that the protein is more than 99% pure

RESULTS

Expression and purification from inclusion bodies of hB7-2V. SDS–PAGE analysis of bacterially expressed hB7-2V revealed the presence of a predominant band migrating with an apparent molecular weight of 13K in the induced lanes (Fig. 1B). The MW of the induced protein was consistent with that of the protein encoded by our DNA construct (12,834). As evident from the uninduced lanes, the protein was also produced, albeit at lower levels, in the absence of IPTG. This basal level production may explain why bacterial cultures typically took twice as long to reach log phase compared to other recombinant proteins we have worked with in this system. To determine if the protein was expressed in the soluble or insoluble fractions, induced cells were lysed by sonication, centrifuged at 15,000*g*, and analyzed by SDS–PAGE (data not shown). This analysis demonstrated that the recombinant protein was exclusively expressed as inclusion bodies. Four liters of bacterial culture finally yielded ~400 mg solubilized inclusion bodies that was determined to be approximately 95% pure by SDS–PAGE analysis (data not shown).

Refolding and purification of hB7-2V. Eight hours after refolding, the protein solution was filtered and 0.8 ml was loaded onto a Superdex G75 gel-filtration column. This revealed a monodisperse peak at ~20 min

(data not shown), consistent with the predicted MW of our recombinant protein and establishing that hB7-2V existed as a monomeric species in solution. Notably, there were no other peaks present and no evidence of soluble, misfolded aggregates, as nothing was observed to run at what corresponded to the void volume time (10 min) for this column. The refolding efficiency was estimated to be ~50%, as the refolding solution contained nearly 50 mg of soluble protein as determined by absorbance at 280 nm based on the extinction coefficient for hB7-2V (data not shown).

During the concentration and buffer exchange (to 10 mM Tris–HCl, 20 mM NaCl, pH 8.0) of the hB7-2V refolding solution, a moderate amount of insoluble aggregates appeared to fall out of solution. After centrifugation and filtration, the protein solution was loaded onto a MonoQ anion-exchange column. The protein eluted at ~150 mM NaCl as a discrete, monodisperse peak (Fig. 1A). There were no impurities in the fractions corresponding to this peak as determined by SDS–PAGE analysis (Fig. 1C). The protein was then concentrated and buffer exchanged to 10 mM Tris–HCl, 20 mM NaCl, pH 8.0, for binding assays and crystallization trials. The final yield of soluble, purified protein was determined to be ~35 mg from the original ~100 mg of solubilized inclusion bodies that had been diluted into 1 liter of refolding buffer.

Binding assays between hB7-2V and recombinant, monomeric human CTLA-4. In order to characterize monomeric CTLA-4, which has been detected on the surfaces of T cells (9, 13), we cloned a corresponding CTLA-4 construct in which the last five amino acids of the extracellular domain were deleted, including Cys122, which is responsible for forming the interchain disulfide within the CTLA-4 homodimer. Monomeric CTLA-4 was produced by a refolding and purification protocol very similar to that for hB7-2V and buffer exchanged to 10 mM Tris–HCl, 20 mM NaCl, pH 8.0. In order to determine whether refolded hB7-2V was active, binding assays with this monomeric form of CTLA-4 were performed. Despite removal of Cys122, there still existed a significant proportion of noncovalent CTLA-4 dimer in equilibrium with monomer as seen by gel filtration (Fig. 2A) and native PAGE (Fig. 2B, lane 1). This distribution between monomer and dimer reappeared when gel-filtration chromatography was performed on fractions corresponding to either monomer or dimer, which suggests a dynamic equilibrium between these two species. The CTLA-4 noncovalent dimer and monomer ran at 16 and 19.5 min, respectively, on gel filtration, consistent with their predicted MWs (26,232 and 13,116, respectively) based on their amino acid sequences. Purified, recombinant hB7-2V ran as one monodisperse peak on gel filtration at 20 min (Fig. 2A) and one discrete band on native gel (Fig. 2B, lane 2).

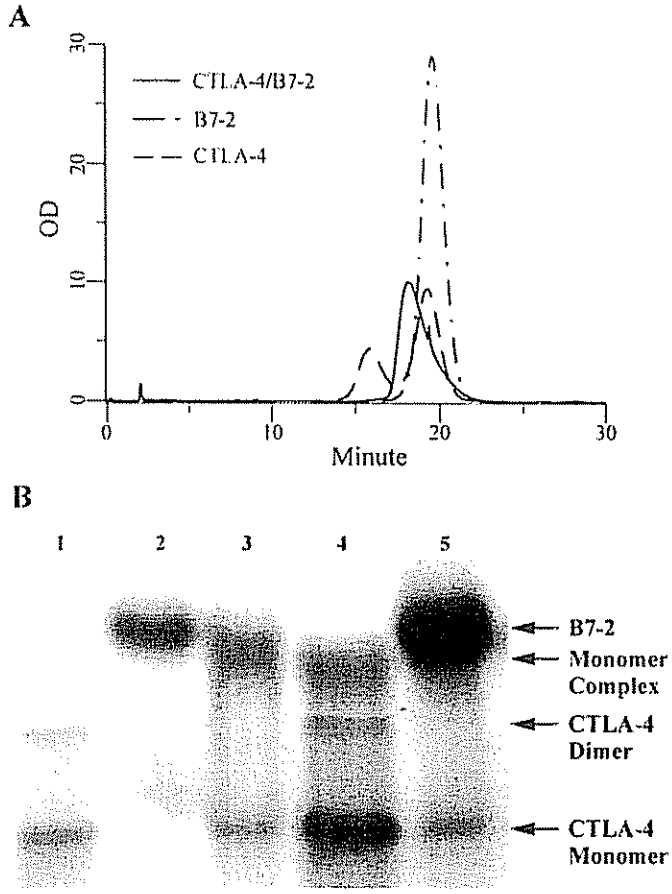

FIG 2. Binding assays between hB7-2V and monomeric human CTLA-4. (A) Elution profiles of hB7-2V, monomeric CTLA-4, and the 1:1 mixture of CTLA-4:hB7-2V from Superdex G-75 gel-filtration column chromatography. The peak at 17.5 min corresponds to the monomeric complex of CTLA-4 and hB7-2V (MW 25,950). (B) Native PAGE analysis of the formation of the CTLA-4/hB7-2V complex. Lane 1, monomeric and noncovalent dimeric CTLA-4, 10 μg. Lane 2, hB7-2V, 10 μg. Lanes 3, 4, and 5 correspond to the mixture of CTLA-4 and hB7-2V at 1:1 (10 μg:10 μg), 4:1 (40 μg:10 μg), and 1:4 (10 μg:40 μg) molar ratios, respectively. (*Note.* Monomeric CTLA-4 and hB72V have approximately equivalent molecular weights.) The formation of the complex is demonstrated by the appearance of a new band in lanes 3, 4, and 5.

These two results together indicate that hB7-2V is exclusively monomeric in solution. Despite having similar MWs, the CTLA-4 monomer migrates much faster than hB7-2V on native gel due to the fact that CTLA-4 contains three additional net negative charges (Fig. 2B, lanes 1 and 2). After incubation of hB7-2V and monomeric CTLA-4 at a 1:1 molar ratio overnight at 4°C, both gel-filtration chromatography and native PAGE demonstrated the formation of a distinct species corresponding to the monomeric CTLA-4/B7-2 complex. On our gel-filtration column, the peak at 17.5 min (Fig. 2A) corresponds to a species larger than both monomeric hB7-2V and monomeric CTLA-4, yet smaller than dimeric CTLA-4, which is consistent with the formation

of a monomeric complex. Despite the fact that the monomeric complex and dimeric CTLA-4 have approximately equivalent MWs, the observation that the monomeric complex adopts a much more compact conformation than dimeric CTLA-4 in the CTLA-4/B7-2 complex structure explains why they do not elute at the same time on gel filtration. We confirmed the presence of both CTLA-4 and hB7-2V in the monomeric complex peak by SDS–PAGE analysis (data not shown). It is interesting to note that while dimeric CTLA-4 has been completely exhausted by addition of hB7-2V (no peak on gel filtration at 16 min), there does not exist a species corresponding to the dimeric complex consisting of the CTLA-4 dimer and two B7 molecules. Consistent with these results, the 1:1 molar ratio mixing of CTLA-4 and B7 resulted in the appearance of only one new band, which corresponds to the monomeric complex observed on gel-filtration chromatography and exhaustion of the dimeric CTLA-4 species on native PAGE (Fig. 2B, lane 3). Despite having approximately twice the MW of hB7-2V, the monomeric complex migrates slightly faster than hB7-2V on native gel, consistent with the fact that CTLA-4/B7 complex has four additional net negative charges. For reasons unknown to us, the monomer complex does not run as a tight band on native gel. Increasing the molar ratio of CTLA4:hB7-2V to 4:1 results in the formation of the expected monomer complex band and exhaustion of the hB7-2V band on native gel (Fig. 2B, lane 4). Reversing the molar ratio of CTLA-4:hB7-2V to 1:4 again results in the disappearance of the CTLA-4 dimer band, while the monomer complex band is obscured by the strong hB7-2V band (Fig. 2B, lane 5).

Binding assays between hB7-2V and recombinant, covalent dimeric human CTLA-4. The bacterially expressed and refolded covalent CTLA-4 homodimer runs as a monodisperse peak at 16 min on a Superdex G75 gel-filtration column, consistent with its predicted MW of 27,268 (Fig. 3A). On native PAGE, CTLA-4 runs as one sharp band (Fig. 3B, lane 1). This is in contrast with the monomeric CTLA-4 construct we described above, which ran as two species on both gel filtration and native PAGE. Here, the interchain disulfide in the covalent CTLA-4 dimer does not allow for dissociation into monomers. On native gel, this covalent CTLA-4 homodimer runs much faster (Fig. 3B, lane 1) than the noncovalent CTLA-4 dimer did (Fig. 2B, lane 1) due to the two additional negative charges contained within the five C-terminal amino acids (CPDSD) present only in the covalent CTLA-4 dimer construct. Incubation of the covalent CTLA-4 homodimer and hB7-2V at a 1:1 molar ratio (based on monomer concentration) resulted in the appearance of a new species on both gel filtration (Fig. 3A) and native gel (Fig. 3B, lane 3). On gel filtration, a new peak appeared at 14.5 min, corresponding to the dimeric CTLA-4/B7-2 complex (some excess hB7-2V is also present). On native gel, a discrete, tight dimeric CTLA-4/B7-2 complex band is apparent, which migrates slower than the hB7-2V band. Increasing the CTLA-4:hB7-2V molar ratio to 4:1 results in the near-complete exhaustion of the hB7-2V band and the appearance of an intense dimer CTLA-4/B7-2 complex band on native gel (Fig. 3B, lane 4). Reversing the CTLA-4:hB7-2V molar ratio to 1:4 results in the predicted near exhaustion of the CTLA-4 dimer band and an intense CTLA-4/B7-2 complex band on native gel (Fig. 3B, lane 5).

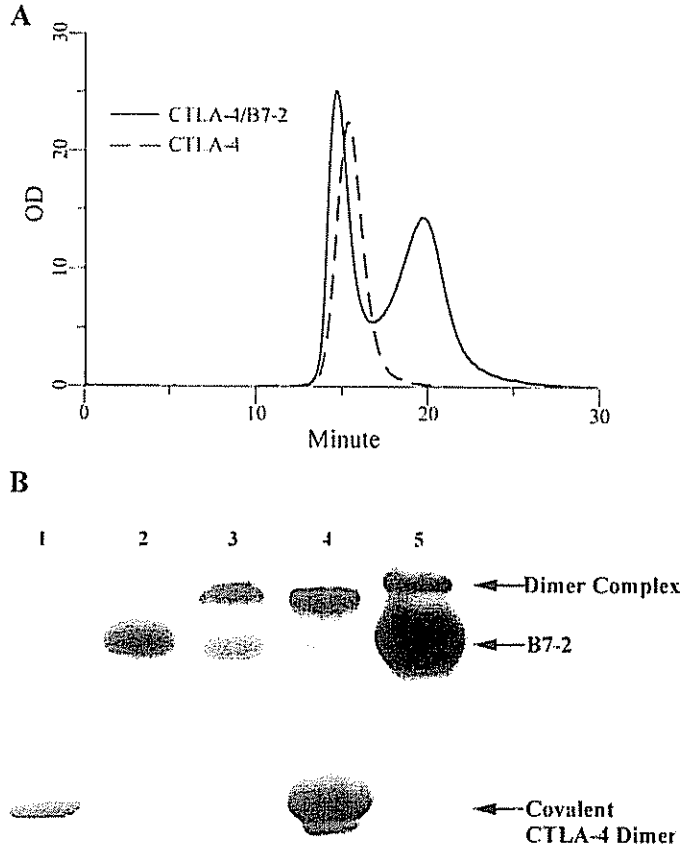

FIG. 3. Binding assays between hB7-2V and disulfide-linked dimeric human CTLA-4. (A) Elution profiles of disulfide-linked CTLA-4 and the 1:1 (based on monomer concentration) mixture of dimeric CTLA-4/hB7-2V from Superdex G-75 gel-filtration column chromatography. The complex between the CTLA-4 dimer and hB7-2V elutes ~0.5 min earlier than the CTLA-4 dimer itself. (B) Native PAGE analysis of the formation of the dimeric CTLA-4/hB7-2V complex. Lane 1, CTLA-4 dimer, 10 μg. Lane 2, B7-2, 10 μg. Lanes 3, 4, and 5 correspond to the mixture of dimeric CTLA-4 and hB7-2V at 1:1 (10 μg:10 μg), 4:1 (40 μg:10 μg) and 1:4 (10 μg:40 μg) molar ratios (based on monomer concentration), respectively. The formation of the complex is demonstrated by the appearance of a new band in lanes 3, 4, and 5.

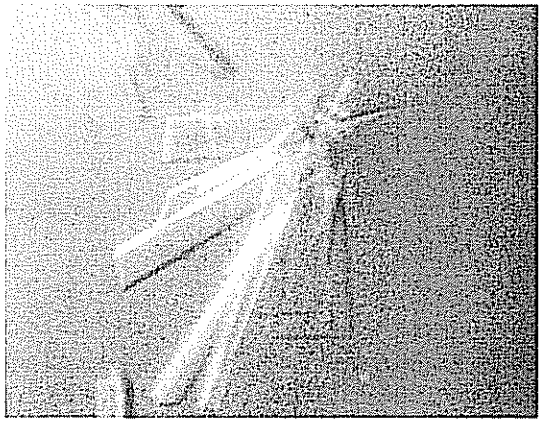

FIG. 4. Native crystals of hB7-2V. Crystals were grown for ~10 days in the refined condition described under Materials and Methods.

Crystallization and X-ray diffraction analysis of the receptor-binding domain of hB7-2V. Plate-like rectangular crystals (1.0 × 0.1 × 0.05 mm) of hB7-2V grew within 10 days at 4°C in 0.1 M NaAc, 0.1 M Tris–HCl, 20% PEG 4000, pH 8.5 (Fig. 4). The corresponding selenomethionyl-substituted protein grew to similar dimensions under the same conditions in the presence of 1 mM DTT. Native and selenomethionyl-substituted crystals diffracted to 2.3 and 2.7 Å, respectively. Analysis of the native data set to 2.3 Å using the HKL suite revealed that the data were highly mosaic (>2.0°) and that the higher resolution shells displayed large merging R factors. On the other hand, the 2.7 Å data set collected for the selenomethionine-substituted crystal exhibited a much lower degree of mosaicity (~1.2°) and had acceptable merging R factors. Diffraction from the selenomethionine crystals was consistent with the orthorhombic space group $P2_12_12$ with unit cell dimensions $a = 56.69$, $b = 63.01$, and $c = 58.61$ Å. Assuming two hB7-2V monomers in the asymmetric unit, the Matthews volume and the solvent content are calculated to be 2.04 $Å^3$ Da^{-1} and 39.21%, respectively. Further analysis of the data and structure solution by the MAD method is in progress.

DISCUSSION

We have described the first method for producing large quantities of a folded, active B7 costimulatory ligand in a bacterial expression system. We have demonstrated that the V-type Ig domain of B7-2 binds both monomeric and dimeric CTLA-4. The recent crystal structure determination of human B7-1 showed that the analogous V-type Ig domain in this molecule was solely responsible for mediating dimerization, further supporting the functional significance of this region of B7 (14, 16). In addition, CTLA-4/B7-2 binding does not require glycosylation, as the recombinant forms of CTLA-4 and B7-2 used in this study were both produced in bacterial expression systems.

Cys122 in CTLA-4 has been previously shown to form an intermolecular disulfide mediating CTLA-4 covalent homodimerization on the T cell surface (12). We have shown by gel filtration and native PAGE analysis that deletion of Cys122 results in a dynamic equilibrium between monomeric and noncovalent dimeric forms of CTLA-4. Interestingly, addition of recombinant hB7-2V to this equilibrium of CTLA-4 monomers and dimers results in the complete disappearance of all noncovalent dimers with no evidence of dimeric CTLA-4/B7-2 complex formation, whereas monomeric CTLA-4/B7-2 complex formation is readily detected, suggesting that the binding of hB7-2V somehow perturbs the dimer interface of noncovalent dimeric CTLA-4. However, the crystal structures of the CTLA-4/B7 complexes have shown that B7 does not directly contact residues in the CTLA-4 dimer interface (15, 16). To explain this apparent paradox, we propose that the binding of B7-2 to dimeric CTLA-4 results in a minor conformational change in CTLA-4 that affects the dimerization interface, destabilizing the noncovalent dimer, finally leading to its dissociation into individual monomer species. Binding of B7-2 to a covalent CTLA-4 homodimer may result in a similar conformational change and perturbation of the dimerization interface but with no resultant dissociation owing to the disulfide linkage. In this case, the conformational change at the dimer interface may provide a mechanism for communication between the two B7-2 binding sites on CTLA-4, that is, the binding of one B7 molecule to the first CTLA-4 subunit may affect the binding of a second B7 molecule to the remaining CTLA-4 subunit, perhaps enhancing (positive cooperativity) or decreasing (negative cooperativity) the affinity of this interaction.

The ability to produce large quantities of a homogeneous and active T cell costimulatory ligand in an efficient, inexpensive manner might be of great utility for modulating the activity of T cells both *in vitro* and *in vivo*. A recent study reported that soluble full-length B7-2 was detectable in human serum at concentrations sufficient to costimulate T cells (24). This study further demonstrated that recombinant soluble B7-2, produced in Sf9 cells, is a functional costimulatory molecule *in vitro* that can induce T cell activation (in combination with an anti-CD3 mAb) and reasoned that soluble B7-2 might play an important role in immune regulation by binding to either CD28 or CTLA-4 *in vivo*. Another report demonstrated that microspheres coated with soluble recombinant B7-1 or B7-2, produced in a mouse myeloma NSO cell line, and peptide–MHC complexes can efficiently activate antigen-specific T cells both *in vitro* and *in vivo* under well-controlled antigenic and costimulatory conditions (30). These studies demonstrate the potential utility of soluble recombinant B7-2

for regulating T cell activity. The methodology we have presented here should facilitate the efforts of scientists and clinicians alike to use this ligand for manipulating T cell responses to pathogens, cancers, and autoantigens. Finally, we have used this technology for the preparation of unliganded, selenomethionine-labeled hB7-2V crystals in addition to the CTLA-4/B7-2 complex crystals.

ACKNOWLEDGMENT

This work was supported by National Institutes for Health Research Grant AI07289, Training Grant CA10702, and Juvenile Diabetes Foundation 1-2000-743.

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