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Crystallization and functional analysis of a soluble deglycosylated form of the human costimulatory molecule B7-1

Simon J. Davis,[a]* Shinji Ikemizu,[b] Alison V. Collins,[a] Janet A. Fennelly,[a] Karl Harlos,[b,c] E. Yvonne Jones[b,c] and David I. Stuart[b,c]

[a]Nuffield Department of Clinical Medicine, The University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, England, [b]Division of Structural Biology, The Henry Wellcome Building for Genomic Medicine, The University of Oxford, Roosevelt Drive, Oxford OX3 7BN, England, and [c]Oxford Centre for Molecular Sciences, The University of Oxford, New Chemistry Laboratory, South Parks Road, Oxford OX1 3QT, England

Correspondence e-mail: sdavis@molbiol.ox.ac.uk

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The interactions of B7-1 with CD28 and CTLA-4 modulate the course of human immune responses, making B7-1 an important target for developing structure-based therapeutics. B7-1 is, however, one of the most heavily glycosylated proteins found at the leukocyte cell surface, complicating the structural analysis of this molecule. Methods for the production, crystallization and selenomethionine labelling of a soluble deglycosylated form of this molecule are described. The protein readily forms both tetragonal plate and bipyramidal crystals belonging to space groups $I4_122$, with unit-cell parameters $a = b = 56.9$, $c = 298.7$ Å, and $P4_122$ (or $P4_322$), with unit-cell parameters $a = b = 89.0$, $c = 261.9$ Å, respectively. The $I4_122$ and primitive crystal forms diffract to 2.7 and 3.5 Å, respectively. Surface plasmon resonance-based assays indicate that the ligand-binding properties of sB7-1 are unaffected by deglycosylation. Since none of the methods relied on any special structural properties of sB7-1, it is proposed that this novel combination of procedures could in principle be adapted to the systematic analysis of many other glycoproteins of structural or functional interest.

1. Introduction

The B7-1(CD80)/B7-2(CD86)/CD28/CTLA-4(CD152) system generates signals important in determining whether or not a T cell is activated or enters an unresponsive state and for terminating ongoing T-cell responses (reviewed by Greenfield *et al.*, 1998). Altering these interactions has profound effects on immune responses in experimental disease models, resulting in B7 proteins being considered important targets for human immunotherapy (see, for example, Guinan *et al.*, 1999).

B7 molecules are transmembrane glycoproteins belonging to the immunoglobulin superfamily (IgSF) that are expressed on antigen-presenting cells (reviewed by Lenschow *et al.*, 1996). Each is composed of two extracellular domains, a membrane distal V-like domain and a membrane proximal C-like IgSF domain, which are heavily glycosylated (at eight sites per molecule). The B7 ligands, CD28 and CTLA-4, are single IgSF domain transmembrane glycoproteins expressed on T-cell surfaces as disulfide-linked homodimers. In contrast to CD28 and B7-2, which are expressed constitutively or are induced very early in immune responses, the expression of CTLA-4 and B7-1 only occurs after considerable delay (reviewed by Lenschow *et al.*, 1996). CD28-dependent costimulation involves the bulk recruitment of cell-surface molecules and kinase-rich rafts to the site of T-cell receptor engagement, thereby favouring signalling (Wülfing & Davis, 1999; Viola *et al.*, 1999), whereas CTLA-4 inhibits signal transduction by recruiting the tyrosine phosphatase SHP-2 to the receptor (Marengere *et al.*, 1996; Lee *et al.*, 1999).

The dual specificities and distinct expression profiles of each member of the costimulatory system have made the investigation of their functional properties very challenging. We have undertaken a structural approach towards understanding the functional properties and behaviour of these molecules. Herein, we describe the crystallization and binding properties of a deglycosylated soluble form of B7-1. The initial native crystals were of very poor quality and we outline the procedures used to improve their diffraction quality.

2. Results and discussion

2.1. Protein expression

The expression of a cDNA construct encoding the extracellular region of B7-1 (residues 1–201 of the mature polypeptide followed by a hexahistidine tag; sB7-1) in Chinese hamster ovary K1 (CHO-K1) cells has been described previously (van der Merwe *et al.*, 1997). In other work, we have shown that when used at concentrations of 1.5 m*M* the glucosidase I inhibitor *N*-butyldeoxynojirimycin (NB-DNJ) can be used to inhibit the processing of the *N*-glycans of glycoproteins expressed in CHO-K1 cells, facilitating their deglycosylation with endoglycosidase H (endo H) and subsequent crystallization (Davis *et al.*,

1995). However, when we attempted to produce deglycosylated sB7-1 in this manner we could only generate large very inefficiently deglycosylated aggregates (data not shown). This suggested that high levels of NB-DNJ may interfere with the folding of sB7-1 in CHO cells, perhaps through effects on interactions with the chaperones calnexin or calreticulin (which are glucosidase I dependent; reviewed by Parodi, 2000). Chen *et al.* (1998) have shown that the glycosylation inhibitor tunicamycin substantially reduces B7-1 expression on K562 transfectants, suggesting that *N*-glycans also facilitate the folding and expression of B7-1 in these cells.

sB7-1 was subsequently expressed in the mutant CHO cell derivative Lec3.2.8.1, a cell line in which protein folding is expected to proceed normally in the endoplasmic reticulum but *N*-glycans are not processed beyond the endo H sensitive $Man_5GlcNAc_2$ intermediate in the Golgi (Stanley, 1981). It has previously been shown that this cell line is useful for preparing glycoproteins that can be readily deglycosylated prior to structural analysis (Davis *et al.*, 1993). In other work, we have established that the inhibitory effects of the Lec3.2.8.1 phenotype and NB-DNJ on *N*-glycan processing to endo H resistant forms are additive and that under these conditions lower concentrations of NB-DNJ can be used, thereby reducing potential folding effects (Butters *et al.*, 1999). Lec3.2.8.1 clones that secreted sB7-1 at high levels (>20 mg l^{-1}) in the presence of 0.5 m*M* NB-DNJ were therefore generated. After purification by metal-chelate chromatography (Ni-NTA agarose; Qiagen Ltd, Crawley, England) and gel-filtration (Sephadex G-100, Pharmacia, Uppsala, Sweden) as described in van der Merwe *et al.* (1997), the protein proved to be highly soluble and very sensitive to endo H (Butters *et al.*, 1999). The deglycosylated endo H treated protein was purified by lectin-affinity chromatography and gel-filtration as described in Davis *et al.* (1995).

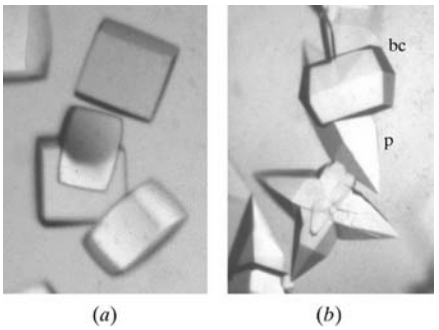

Figure 1
Crystals of human sB7-1. (*a*) Initial crystals of deglycosylated sB7-1 grown prior to removal of the hexahistidine tag. (*b*) Body-centred (bc) and primitive (p) crystals of SeMet-labelled sB7-1 used for multiple wavelength anomalous dispersion data collection at the ESRF.

2.2. Crystallization and preliminary X-ray analysis

Initial conditions for crystallization by vapour diffusion were screened using a sparse-matrix crystallization screening kit (Hampton Research, Laguna Niguel, California, USA). The protein, concentrated to 14 mg ml^{-1} in HEPES buffered saline (10 m*M* HEPES, 140 m*M* NaCl, 0.05% NaN_3 pH 7.4), was mixed with equal volumes of precipitant in 2–4 µl sitting drops on Microbridges (Hampton Research) at 293 K. Crystals or microcrystals formed under 15 of the 98 conditions tested; representative examples of the largest most promising crystals, *i.e.* tetragonal plates grown in 10–28% PEG 400, 0.1 *M* Na HEPES pH 7.5, 0.2 *M* $CaCl_2$, are shown in Fig. 1(*a*).

Of the crystals that could be tested, none diffracted beyond 8 Å resolution in-house using a Rigaku RU-200 generator and 18 and 30 cm MAR Research imaging plates (Norderstedt, Germany) for data collection. However, a substantial improvement in diffraction was obtained when crystals were grown from protein treated with carboxypeptidase A to remove the hexahistidine tag (van der Merwe *et al.*, 1997). In addition to the tetragonal plates, the detagged protein yielded a second, tetragonal bipyramidal crystal form under the same conditions. Initial characterization in-house indicated that the square plates belong to the $I4_122$ space group, with unit-cell parameters $a = b = 56.9$, $c = 298.7$ Å, and that the bipyramids belong to the $P4_122$ or $P4_322$ space groups, with unit-cell parameters $a = b = 89.0$, $c = 261.9$ Å. The new crystals still diffracted weakly (to ∼4 Å resolution), but larger better diffracting crystals were subsequently grown by increasing the protein concentration to 21 mg ml^{-1}. The largest body-centred crystals diffracted to 2.7 Å resolution at beamline 14.2 of the Daresbury synchroton and the primitive-lattice crystals diffracted to 3.5 Å at beamline ID2 of the European Synchrotron Radiation Facility (ESRF). Diffraction data sets collected for both crystal forms were processed using the *HKL* program suite (Otwinowski & Minor, 1997), giving $R_{\rm merge}$ values of 12% to 2.7 Å for the body-centred crystals and 10.8% to 3.5 Å for the primitive crystals. The overall and outer-shell $I/\sigma(I)$ values for the body-centred and primitive crystals were 20.6 and 5.5 (2.8–2.7 Å) and 11.6 and 2.8 (3.63–3.5 Å), respectively.

2.3. Selenomethionine labelling of sB7-1

Molecular-replacement methods utlilizing the programs *AMoRe* (Navaza, 1994), *X-PLOR* (Brünger, 1992) and *GLRF* (Tong & Rossmann, 1997) were initially undertaken in an attempt to solve the structure of the $I4_122$ crystal form. Search models included rat CD2 domain 1 (d1), human CD2d1, human CD4d1, human CD8 and a model consisting of a series of superimposed V-set IgSF domains with or without loop regions. All of these trials were unsuccessful. An in-house search for heavy-atom derivatives was also initiated, but this was problematic owing to crystal anisotropy and non-isomorphism between native and derivatized crystals. The high levels of sB7-1 expression obtained using the Lec3.2.8.1 cells, along with the relatively high incidence of methionine residues in the protein sequence (six of 201 residues), made selenomethionine (Se-Met) labelling of the protein followed by analysis using multiple anomalous dispersion (MAD) an attractive phasing strategy.

Following the method of May *et al.* (1997), the cell line secreting sB7-1 was grown to confluence in four Cell Factories (Nunc, Roskilde, Denmark), after which sodium butyrate was added to the cultures to a final concentration of 2 m*M*. 2 d later, the medium was removed and each of the Factories was rinsed with PBS prior to the addition of 0.5 l of methionine-free Dulbecco's modification of Eagle's medium containing 30 mg l^{-1} L-selenomethionine, 10% FCS, 0.5 m*M* NB-DNJ and 2 m*M* sodium butyrate. After one week at 310 K, the supernatants were harvested, centrifuged at 10 000*g* for 30 min to remove cellular debris, adjusted to pH 8.0 and diluted threefold with PBS pH 8.0, prior to purification of the SeMet-labelled sB7-1 by metal-chelate chromatography and gel filtration. The 2 l culture yielded 16.3 mg of monomeric SeMet-labelled protein. Proton-induced X-ray emission spectroscopy indicated that 60% of the methionine residues were substituted with SeMet (data not shown). However, the solubility of the deglycosylated protein was markedly reduced by substitution with SeMet to the extent that crystal nucleation was initiated simply by concentrating the protein to >21 mg ml^{-1} in HEPES-buffered saline. Under these conditions, the protein formed masses of very small crystals or microcrystalline precipitates. However, when the

protein concentration was kept below 16 mg ml^{-1} in PEG 400, 0.1 *M* Na HEPES pH 7.5, 0.2 *M* $CaCl_2$, the SeMet-labelled sB7-1 formed both body-centred and primitive crystals that were similar to those formed by the native protein and were sufficiently large for data collection. Examples of these crystals are shown in Fig. 1(*b*). The SeMet-labelled body-centred crystals diffracted to 3 Å at beamline BM14 of the ESRF, where highly redundant MAD data sets were collected for detailed structural analysis (Ikemizu *et al.*, 2000).

2.4. BIAcore analysis of the binding properties of the deglycosylated protein

Hollsberg *et al.* (1997) have described a hypoglycosylated form of B7-2 expressed on T cells that appears to have reduced ligand-binding activity. It has also been argued (Wyss *et al.*, 1995), albeit controversially (Davis & van der Merwe, 1996), that the stabilities of other IgSF molecules may in some instances be glycosylation-dependent. For these reasons, it was necessary to establish that the deglycosylated sB7-1 used in this study was fully active. We have previously used surface plasmon resonance-based binding assays, as implemented by BIAcore (Stevenage, Hertfordshire, England), to characterize the interactions of CHO cell-expressed sB7-1 with its ligands CD28 and CTLA-4 (van der Merwe *et al.*, 1997). In similar assays, deglycosylated sB7-1 prepared from Lec3.2.8.1 cell cultures bound to immobilized CD28 and CTLA-4 with both the fast kinetics (data not shown) and affinities (2 and 0.4 µ*M*, respectively; Fig. 2) characteristic of the fully glycosylated CHO cell-derived protein (4 and 0.4 µ*M*; van der Merwe *et al.*, 1997). These observations suggest that the ligand-binding properties and presumably the structure of sB7-1 are largely unaffected by deglycosylation.

3. Conclusions

B7-1 represents an attractive target for the structure-based design of therapeutics useful for treating immune-system-related pathologies. The folding properties and extensive glycosylation of sB7-1 initially constituted significant obstacles to the crystallization and structural analysis of this molecule. Because the *N*-linked glycans have little or no apparent role in sustaining the overall structural integrity of the protein once folding is complete, however, we were able to grow SeMet-labelled crystals of fully active sB7-1 that were of sufficient quality for detailed and rapid structural analysis.

The sB7-1 crystals do not yet diffract to very high resolution, probably because of variation in crystal packing at the major lattice contact (S. Ikemizu *et al.*, unpublished). However, the crystal structure of sB7-1 has been determined to 3 Å resolution using the initial MAD data (Ikemizu *et al.*, 2000), providing a useful basis for initiating the design of bioactive inhibitors of B7-1–ligand interactions. Using new highly redundant native data sets collected at Daresbury station 14.2, the structure has recently been refined to 2.7 Å resolution (Ikemizu *et al.*, unpublished data).

There is considerable interest in the systematic structural analysis of the protein products of new genes identified in the course of high-throughput genome sequencing. Secreted and cell-surface glycoproteins form a subset of proteins that are likely to be of considerable interest in this context. This class of proteins presents special problems for conventional high-throughput methods, however, because their expression in bacteria is unlikely to be straightforward given the requirement for disulfide-bond formation and/or glycosylation in many instances. On the other hand, in our experience, most monomeric glycoproteins can be readily expressed in a fully active state at relatively high yields using mammalian expression systems. We note that none of our methods for the production and analysis of the sB7-1 crystals relied on any special properties of sB7-1. We propose, therefore, that the novel combination of procedures we have employed, namely the production of SeMet-labelled protein with a generic (hexahistidine) tag for purification, coupled with a general method for deglycosylating proteins after folding is successfully completed (secretion from Lec3.2.8.1 cells), could in principle be adapted to the systematic high-throughput analysis of many other glycoproteins of structural or functional interest.

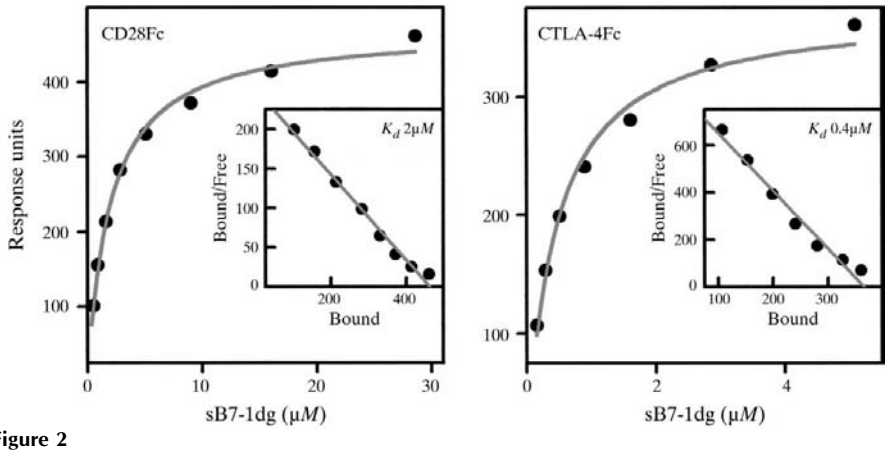

Figure 2
Equilibrium binding analysis of deglycosylated (dg) sB7-1 binding to CD28 and CTLA-4 expressed as chimeras with human IgG Fc (referred to as CD28Fc and CTLA-4Fc, respectively). sB7-1dg, at a range of concentrations, *i.e.* 0.5–28.6 and 0.2–5.1 µ*M*, was injected over immobilized CD28Fc (7100 RU) or CTLA-4Fc (1550 RU), respectively. The plots represent non-linear fits of the Langmuir binding isotherm to the binding data. Scatchard plots of the data are also shown (inset). Details of the preparation of CTLA-4Fc and CD28Fc are given in van der Merwe *et al.* (1997).

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