Protein Engineering, Design & Selection vol. 20 no. 2 pp. 91–98, 2007
Published online February 8, 2007 doi:10.1093/protein/gzl058

Monovalent antibody scFv fragments selected to modulate T-cell activation by inhibition of CD86–CD28 interaction

Reto Kolly[1], Michael A. Thiel[2], Thomas Herrmann[3] and Andreas Plückthun[1,4]

[1]Department of Biochemistry, University of Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland, [2]Department of Ophthalmology, University of Zürich, Frauenklinikstr. 24, CH-8091 Zürich, Switzerland. [3]Institute of Virology and Immunobiology, Julius-Maximilians-Universität Würzburg, Versbacher Strasse 7, D-97078 Würzburg, Germany

[4]To whom correspondence should be addressed.
E-mail: plueckthun@bioc.unizh.ch

Beside the interaction of the antigen-presenting major histocompatibility complex with the T-cell receptor, a co-stimulatory signal is required for T-cell activation in an immune response. To reduce immune-mediated graft rejection in corneal transplantation, where topical application of drugs in ointments or eye-drops may be possible, we selected single-chain antibody fragments (scFv) with binding affinity to rat CD86 (B7.2) that inhibit the co-stimulatory signal. We produced the IgV-like domain of rat CD86 as a fusion protein in *Escherichia coli* by refolding from inclusion bodies. This protein was used as a target for phage display selection of scFv from HuCAL-1®, a fully artificial human antibody library. Selected binding molecules were shown to specifically bind to rat CD86 and inhibit the interaction of CD86 with CD28 and CTLA4 (CD152) in flow cytometry experiments. In an assay for CD86-dependent co-stimulation, the selected scFv fragment successfully inhibited the proliferation of T-cells induced by CD86-expressing P815 cells.
Keywords: antibody library/CD28/CD86/co-stimulation/phage display

Introduction

Upon the interaction of antigen-presenting cells (APC) with helper and cytotoxic T-cells, the presentation of the peptide antigen on the MHC molecule to the T-cell receptor flanked by CD4 or CD8, respectively, plays the primary role for T-cell activation (first signal). But a second signal, mainly regulated by the interaction of the surface proteins CD80 (B7.1) and CD86 (B7.2) on the APC and CD28 and CTLA4 (CD152) on the T-cell, directs the outcome of the interaction either towards tolerance or an immune response to the presented antigen. Although additional members of the B7 family have been discovered (Coyle and Gutierrez-Ramos, 2001; Carreno and Collins, 2002; Liang and Sha, 2002), CD80 and CD86 are still considered the most important molecules in the initiation and maintenance of the immune response.

Upon interaction of CD80 or CD86 with CD28, an activating signal is produced, which leads to IL-2 production and entry of the T-cell into the cell cycle, followed by sustained proliferation (Shapiro *et al.*, 1997; Bonnevier *et al.*, 2006; Colombetti *et al.*, 2006). In contrast, interaction of CD80 or CD86 with CTLA4 results in a negative signal inhibiting IL-2 production and termination of T-cell responses (Leach *et al.*, 1996; Saito, 1998). Although CD86 is constitutively expressed and further upregulated upon activation, CD80 is only induced after T-cell activation (Bugeon and Dallman, 2000). On the T-cell surface, CD28 is constitutively present, whereas CTLA4 is upregulated only upon activation of the T-cell (Linsley and Ledbetter, 1993; Lenschow *et al.*, 1996). Therefore, the interaction between CD86 and CD28 is thought to be the initiating co-stimulatory signal (Bugeon and Dallman, 2000). Recent studies have sought to dissect the role of CD80 and CD86, respectively, and it appeared that they do indeed mediate distinct signals in previously activated T-cells (Rogers *et al.*, 2005). Even though CD80 and CD86 are both important, it is the molecular partner that CD86 interacts with (CD28 or CTLA4) that seems to be particularly decisive for the fate of the immune reaction.

Inhibition of this first activating interaction between CD86 and CD28 is expected to lead to a reduced activation of T-cells. Such an effect would be of great importance in transplantation medicine, where the risk of graft tissue rejection can thereby be reduced.

Focusing in particular on corneal transplantation, where the antigenic response is mostly triggered by foreign MHC molecules from the donor tissue, different antibodies targeting T-cell activation have been studied. Inhibition of graft rejection could be achieved with antibodies targeting mainly T-cell determinants or co-stimulatory molecules. In the inhibition of co-stimulatory signaling in animal models, binding of CD80 and CD86 to CD28 was impaired by either systemically administering anti-CD80 and anti-CD86 monoclonal antibodies or by CTLA4–Fc, a fusion protein of the extracellular domain of CTLA4 to the Fc part of an IgG molecule (Kagaya *et al.*, 2002; Thiel *et al.*, 2005). Although the topical application of CTLA4–Fc did not show efficacy, systemic administration or treatment of donor cornea with CTLA4–Fc before transplantation showed a prolongation of graft survival (Hoffmann *et al.*, 1997; Gebhardt *et al.*, 1999).

Corneal transplantations are uniquely suited to test such approaches of inhibiting graft rejections as the special location of the cornea on the body surface allows topical administration of drugs via ointment or eye drops. This unique mode of administration of drugs possible in the eye allows high concentration of effector molecules to be present while avoiding systemic side effects. It was previously shown that proteins of up to 60 kDa molecular weight are able to diffuse through the cornea tissue (Thiel *et al.*, 2002).

We selected single-chain antibody fragments (scFv) for binding to rat CD86, which have a molecular weight of ~30 kDa, from HuCAL-1 (Knappik *et al.*, 2000) (MorphoSys AG), a library of synthetic human antibody fragments. As the binding target, one extracellular domain of

rat CD86, which interacts with CD28, was expressed in *Escherichia coli* and refolded from inclusion bodies to a soluble, active protein. The scFv fragments were shown to selectively bind to CD86 and functionally inhibit its interaction with CD28 and CTLA4, and indeed inhibited the proliferation of T-cells in the presence of cells expressing CD86 on their surface.

Materials and methods

Protein expression and purification

The IgV-like domain of rat CD86 was expressed as a fusion protein, consisting of an N-terminal Avi-tag (sequence GLNDIFEAQKIEWHE) (Cull and Schatz, 2000), the N1-domain of the M13 phage protein g3p (aa 1–81), rat CD86 (aa 2–110) and a C-terminal His_6-tag. cDNA encoding the IgV-like domain of rat CD86 was inserted into the vector pAT231, a derivative of the expression vector pAT222 (Binz *et al.*, 2004) containing the sequence encoding the N1-domain. As a control, the protein without the CD86 domain was produced.

Fusion proteins were expressed as inclusion bodies in BL21 (DE3) pREP4 *E. coli* (Stüber *et al.*, 1990; Studier *et al.*, 1990), also containing the pBirAcm biotinylation plasmid (Cull and Schatz, 2000). Cells were grown at 37°C in 2xYT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter). Thirty minutes after addition of 12 mg/l biotin at $OD_{600} = 0.5$, bacteria were induced with 1 mM IPTG and the culture was incubated for 240 min at 37°C. Cells were collected by centrifugation, resuspended in 10 mM Tris–HCl, 2 mM $MgCl_2$, pH 8, and after addition of a spatula tip of DNAseI and lysozyme each, cells were lysed in a French pressure cell (Aminco French Press). Inclusion bodies were collected by centrifugation and washed three times with 500 mM urea, 100 mM Tris–HCl, pH 8, 0.5% Triton X-100. The cell pellet was suspended in 10 mM Tris–HCl, 2 mM $MgCl_2$, pH 8. Inclusion bodies were then solubilized with 8 M urea, 200 mM Tris–HCl, 50 mM DTT, pH 8.6, and after centrifugation dialyzed against 8 M urea, 200 mM Tris–HCl, pH 8.6. Refolding was performed by rapid dilution into 800 mM arginine, 200 mM Tris, 0.5 mM 6-amino-*n*-caproic acid, 0.5 mM benzamidine hydrochloride, 0.2 mM reduced glutathione and 1 mM glutathione disulfide, pH 9. Refolded protein was concentrated by ultra-filtration and dialyzed against 50 mM NaH_2PO_4, 300 mM NaCl, pH 8. Proteins were purified with a bench top Ni-NTA agarose column (Qiagen) according to the manufacturer's protocols. Eluates were dialyzed against 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.

Selected scFv fragments were expressed in the periplasm of *E. coli* SB536 cells. Pools of selected scFv-encoding sequences were subcloned via restriction sites *Xba*I and *Eco*RI into the expression plasmid pMX7, and *E. coli* SB536 cells were transformed with the constructed vector. Bacteria were grown at 37°C in 2xYT medium containing 0.1% glucose and 34 mg/l chloramphenicol. At $OD_{600} = 0.5$ cultures were induced with 1 mM IPTG.

For small-scale expressions, cultures were incubated for 5 h at 30°C. For periplasmic extracts, cells were collected by centrifugation and incubated overnight in 300 mM boric acid, 150 mM NaCl, 2 mM EDTA, pH 8, at 4°C. After centrifugation, the supernatant was used for enzyme linked immuno-sorbent assay (ELISA) screening.

For large-scale expression of scFv fragments, cultures were incubated for 20 h at 22°C. Bacteria were collected by centrifugation and resuspended in 50 mM NaH_2PO_4, 300 mM NaCl, pH 8. After addition of a spatula tip of DNAseI and 2 mM $MgCl_2$, bacteria were lysed in a French pressure cell. The lysate was filtered and purified on Protino Ni-2000 IMAC column (Macherey-Nagel) or Ni-NTA agarose (Qiagen), washing with 16 column volumes of 50 mM NaH_2PO_4, 300 mM NaCl, pH 8; 12 column volumes of 50 mM NaH_2PO_4, 900 mM NaCl, pH 8; 16 column volumes of 50 mM NaH_2PO_4, 300 mM NaCl, 0.1% Triton X-100, pH 8; and 8 column volumes of 50 mM NaH_2PO_4, 300 mM NaCl, pH 8. Eluates were concentrated by ultra-centrifugation and buffer-exchanged to PBS using Micro BioSpin P-6 columns (BioRad). For proliferation assays, samples were additionally purified on Detoxi-Gel endotoxin removal columns (Pierce) and eluted with PBS. When stored at 4°C under sterile conditions, purified scFv fragments maintained unchanged binding activity for more than 3 months.

Phage display

Phage selections were performed by incubating 33 pmol of biotinylated antigen with 0.33 pmol of phages in 100 μl PBS 0.5% BSA for 1 h at 4°C. The complexes were captured with 1 mg of BSA-blocked streptavidin magnetic particles (Roche) and washed 10 times with PBS 0.5% BSA. Bound phages were eluted with 100 mM glycine, pH 2.2, and neutralized with the same volume of 1 M Tris, pH 8. *E. coli* TG1 cells were infected with eluted phages and plated on LB agar plates containing 1% glucose and 34 mg/l chloramphenicol. The plates were incubated overnight at 30°C, and bacteria were scraped off to inoculate 2xYT medium containing 1% glucose and 34 mg/l chloramphenicol. The culture was incubated at 37°C and at $OD_{600} = 0.5$ the phage library was rescued by infection with VCS M13 helper phage (Stratagene). The bacteria were harvested by centrifugation and resuspended in 2xYT medium containing 30 mg/l kanamycin, 34 mg/l chloramphenicol, 0.1 mM IPTG and grown overnight at 30°C. Phages were precipitated from the culture supernatant by addition of polyethylene glycol PEG-6000 (3.3% final concentration), NaCl (0.4 M final concentration). Phages were resuspended in H_2O, precipitated by addition of polyethylene glycol PEG-6000 (3.3% final concentration), NaCl (0.4 M final concentration) and resuspended in PBS.

Pools of selected scFv-encoding sequences were subcloned via restriction sites *Xba*I and *Eco*RI into the expression plasmid pMX7-FH (Knappik *et al.*, 2000) and *E. coli* SB536 cells were transformed with the generated plasmid.

Enzyme linked immuno-sorbent assay

Pools of selected scFv fragments were screened by ELISA experiments performed on immobilized antigen. Maxisorp plates were coated overnight with neutravidin (Pierce, 66 nM in PBS) and blocked for 1 h with PBS containing 0.5% BSA. Biotinylated antigen was added at 10 μM in PBS containing 0.1% BSA. Alternatively, non-biotinylated antigen was directly immobilized to Maxisorp plates by incubating antigen at 10 nM in PBS for 1 h at room temperature, followed by blocking with PBS containing 0.5% BSA for 1 h at

room temperature. Crude cell extracts or purified antibody fragments diluted in PBS containing 0.1% BSA were added and incubated at room temperature. Bound scFv fragments were incubated with an anti-FLAG M2 antibody—HRP (horseradish peroxidase) conjugate (Sigma) and detected by a subsequent color reaction (soluble BM blue POD substrate, Roche Diagnostics). Absorbance was measured at 360 and 492 nm (reference wavelength) on a HTS 7000 Plus plate reader (Perkin–Elmer).

Flow cytometry measurements (FCM)

Binding and inhibitory function of selected scFv fragments to P815 mouse mastocytoma cells expressing rat CD86 was analyzed in flow cytometry experiments. Cells were gratefully obtained from Dr. Hideo Yagita, Juntendo University, Tokyo, and were grown in cell culture in RPMI-1640 medium with glutamine (Invitrogen), 10% fetal calf serum (FCS) (Invitrogen), 0.5 mg/ml Geneticin (Invitrogen). Adherent and non-adherent P815 cells were harvested and 1.3×10^6 cells were seeded in 5 ml polystyrene tubes (Falcon). Cells were washed 2 times with PBS, 0.05% sodium azide and resuspended in PBS, 0.05% sodium azide, containing scFv and/or human CD28–mouse Fc (Ancell) or mouse CTLA4–mouse Fc fusion proteins (Chimerigen) at various concentrations and incubated for 60 min at 4°C. P815 cells were washed 2 times with PBS, 0.05% sodium azide. For detecting the binding of scFv fragments, P815 cells were resuspended in PBS, 0.05% sodium azide, containing 2 μg/ml of anti-PentaHis antibody—Alexa Fluor 488 dye conjugate (Qiagen). For binding detection of mouse Fc-containing molecules, P815 cells were resuspended in PBS, 0.05% sodium azide, containing 20 μg/ml of anti-mouse Fc antibody—Alexa Fluor 488 dye conjugate (Invitrogen). After incubation for 60 min at 4°C, cells were washed with PBS, 0.05% sodium azide, resuspended in Cytofix buffer (BD PharMingen) and analyzed on a Becton Dickinson FACSCalibur instrument.

Co-stimulation assay

The inhibitory function of selected scFv fragments on the activation of T-cells by interaction with CD86 or CD80 was analyzed in a modified version of a previously described co-stimulation assay (Maeda *et al.*, 1997), in which blockade of co-stimulatory signals provided by P815 mastocytoma cells stably transduced with CD80 or CD86, respectively, leads to a reduction of proliferation of the rat T-cells, which is measured by a incorporation of ^{3}H-labeled thymidine.

Lymph nodes were taken from a Lewis (LEW/Crl) rat (Charles River, Sulzfeld, Germany) suspended in balanced salt solution (BSS) (3.2 g/l NaCl, 0.2 g/l KCl, 0.09 g/l $CaCl_2$, 0.1 g/l $MgCl_2$, 0.1 g/l $MgSO_4$, 0.12 g/l NaH_2PO_4, 0.03 g/l KH_2PO_4, 0.5 g/l glucose, 0.004 g/l phenol red) with 5% FCS and meshed through a 70 μm sieve (BD Falcon). Cells were washed twice with BSS 5% FCS and resuspended in BSS 5% FCS. T-cells were enriched by passage through a nylon wool column and washed sequentially with BSS and RPMI-1640-SC medium (RPMI-1640 medium supplemented with 5% heat-inactivated FCS, 1 mM sodium pyruvate, 1× MEM non-essential amino acids, 100 U/ml penicillin, 100 U/ml streptomycin, 25 μM β-mercaptoethanol, 290 mM glutamine). P815 mouse mastocytoma cells were cultured in RPMI-1640-SC at 37°C (5% CO_2) in a humidified atmosphere. Non-adherent cells were collected by centrifugation, washed twice with BSS and resuspended in BSS containing 100 μg/ml mitomycin C (Sigma). P815 cells were incubated for 40 min at 37°C and washed three times with RPMI-1640-SC.

In a total volume of 200 μl RPMI-1640-SC, 10^5 purified T-cells were co-cultured with 10^4 mitomycin C-treated P815 cells in 96-well microtiter plates with round bottom wells (Cellstar suspension culture plates, Greiner Bio-One). Anti-rat CD3 antibody G4.18 (PharMingen) was added at 2 μg/ml. Inhibitory scFv fragments were added at 5 μg/ml, mouse CTLA4–Fc was added at 2.5 μg/ml.

The mammalian cells were grown for 48 h at 37°C (5% CO_2). Then 25 μl of a 0.5 μCi/ml solution of ^{3}H-labeled thymidine (Amersham-Buchler, Braunschweig, Germany) in RPMI-1640-SC was added and incubation was continued for 18 h. Cells were harvested on a TOMTEC harvester and after addition of scintillation fluid, ^{3}H emission was detected on a Betawin workstation (Perkin–Elmer).

Results

Expression of rat CD86 IgV-like domain as antigen

The interaction between CD86 and CD28 is thought to be the co-stimulatory signal that initiates the activation of T-cells by APC (Bugeon and Dallman, 2000). To obtain binding molecules inhibiting this interaction, phage selection experiments against the protein CD86 were performed. Because inhibitory molecules resulting from these selections were to be used in a rat model system, CD86 from rat was chosen as the antigen.

The IgV-like extracellular domain of CD86 has been shown to be sufficient for the activating interaction with CD28 (Peach *et al.*, 1995; Rennert *et al.*, 1997). Therefore, only this domain was expressed for the use as an antigen. In addition, non-glycosylated CD86 had previously been shown to retain the ability of CD28 activation (Zhang *et al.*, 2002), and this allowed *E. coli* to be used as an expression system for this antigen.

A fusion protein termed N1–CD86 was constructed, consisting of amino acids 2–110 of the mature rat CD86 sequence fused at the N-terminus to a biotinylatable avi-tag and the N1-domain of the phage M13 protein g3p and at the C-terminus to a His_6-tag (Fig. 1). The N1 sequence was added to improve inclusion body formation of the expressed protein in *E. coli* (Lubkowski *et al.*, 1998; Frisch *et al.*, 2003), based on its low solubility when expressed in the cytoplasm.

CD86 constructs were expressed and enzymatically biotinylated in *E. coli*. Proteins were refolded with a redox shuffle applying a strategy previously used successfully for refolding of antibody scFv fragments (Ge *et al.*, 1995; Proba *et al.*, 1997). After purification under native conditions on Ni-NTA columns, 13 mg of refolded soluble protein was obtained per liter of cell culture.

As determined by SDS–PAGE and analytical gel filtration on a S-200 Superdex column, there were no detectable impurities in this preparation. Functionality of refolded N1–CD86 was verified by ELISA experiments analyzing the binding of the rat CD86 fusion construct to immobilized human CTLA4–Fc (data not shown).

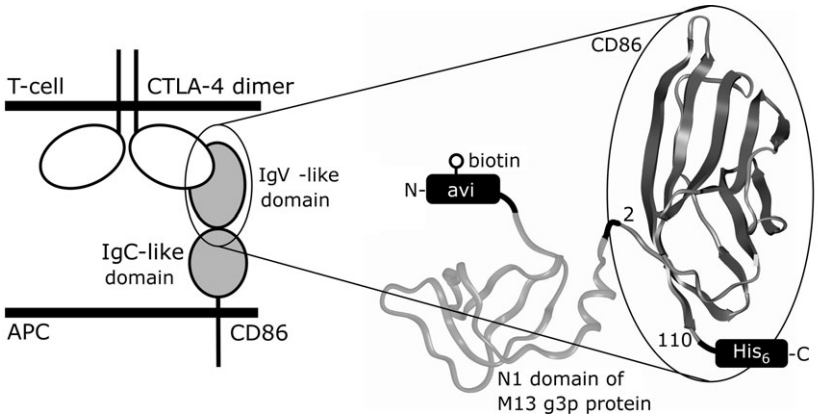

Fig. 1. Schematic representation of the CD86 fusion construct used for selection and evaluation of binding proteins. The IgV domain of rat CD86 interacting with CD28 or CTLA4 was expressed as a fusion construct containing a biotinylatable avi-tag for immobilization and the N1 domain from phage M13 g3p on the N-terminus. A His_6 tag was added to the C-terminus for protein purification. PDB data used: human CD86, 1I85 (Schwartz *et al.*, 2001); N1 domain, 1G3P (Lubkowski *et al.*, 1998).

Applying the same expression procedure, production of the fusion partner alone (to be used as a control), avi-N1–His_6, yielded 18 mg protein per liter of *E. coli* culture.

Analysis of refolded avi-N1–CD86–His_6 and avi-N1–His_6 protein after >3 months at 4°C by gel filtration and ELISA experiments showed the proteins to be stable over this period of time.

Phage display, ELISA analysis

To select scFv fragments binding to CD86, we performed phage display with HuCAL-1 (MorphoSys AG), a fully synthetic library of human scFv fragment sequences containing a diversity of 2×10^9 members (Knappik *et al.*, 2000). M13 phages presenting the HuCAL-1 scFv library as a fusion to the CT domain of g3p coat protein were selected for binding to soluble biotinylated N1–CD86. After the fourth and fifth round of phage display, pools of selected scFv-encoding sequences were inserted into the expression vector pMX7_FH. Single clones were expressed and crude cell extracts were analyzed by ELISA for specific antigen binding activity.

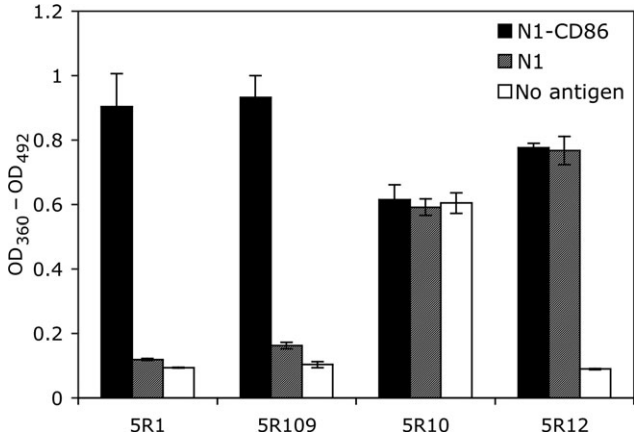

Fig. 2. ELISA analysis. Binding of phage display-selected scFv fragments to refolded rat CD86. The CD86-specific scFv fragments 5R1 and 5R109 show binding to N1–CD86, but not to N1 alone. The weak unspecific binder 5R10 binds to wells with or without immobilized proteins used for selection. ScFv fragment 5R12 binds specifically to both N1–CD86 and N1, and is therefore expected to recognize either of the fusion partners, i.e., avi-tag, N1 or His_6-tag. Data are presented as mean ± SE of two replicates.

We analyzed 16 individual clones from the fourth selection round and 33 clones of the fifth selection round for binding to the N1–CD86 fusion molecule and also tested them for binding to the N1 protein alone. None of the 16 clones from the fourth round showed specific binding to either molecule. After the fifth selection round, however, four different antibody fragments had been selected, two showing specificity to rat CD86-IgV (5R1 and 5R109), one specific to the N1 fusion partner (5R12) and one being a non-specific weak affinity binder (5R10) (Fig. 2).

To test whether the selected scFv fragments might not only bind CD86 from rat but also from other organisms, we performed cross-specificity ELISA experiments. We immobilized 6 pmol each of rat CD86, mouse CD86 and human CD86 fused to human Fc on microtiter plates and tested for binding of purified scFv fragments. Both scFv fragments originating from selection against rat CD86 (5R109 and 5R1) indeed showed species specificity for the rat protein. Interestingly, the same properties were observed for the commercially available anti-rat CD86 monoclonal antibody 24F (Maeda *et al.*, 1997) (Fig. 3). This demonstrates the importance in species-specific selections, even though the extracellular amino acid sequence of mouse CD86 is 78.8% identical and that of human CD86 is

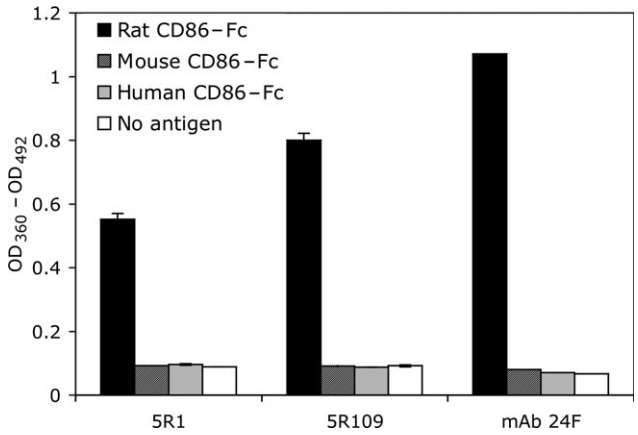

Fig. 3. ELISA analysis. Species specificity of selected scFv fragments. The CD86-specific scFv fragments 5R1 and 5R109 as well as the monoclonal anti-rat CD86 antibody 24F are all specific for rat CD86–Fc fusion protein. Data are presented as mean ± SE of two replicates.

57.9% identical to that of rat CD86 (Maeda *et al.*, 1997). The non-specific binder 5R10 and the N1-specific binder 5R12 showed unspecific or no binding, respectively, to the various CD86–Fc fusions (data not shown).

Inhibitory binding of selected scFv fragments to rat CD86 expressed on mammalian cells

Mouse mastocytoma P815 cells, stably transfected with a BCMGS-neo vector encoding rat CD86 cDNA and thus constitutively expressing this protein on its surface, were grown in cell culture (Maeda *et al.*, 1997). Binding of the different purified scFv fragments to the cell surface was analyzed by flow cytometry at identical scFv concentrations (3.3 μM). The CD86-specific scFv 5R109 produced the highest mean fluorescence intensity (MFI). scFv fragments 5R1 (CD86 specific) and 5R10 (non-specifically adhering scFv) gave intermediate signals, whereas the MFI for the unspecific scFv 5R12 was at the same level as the background signal (Fig. 4A).

Quantitative binding curves of scFvs 5R1 and 5R109 to rat CD86-expressing P815 cells were measured over a concentration range from 640 fM to 10 μM (Fig. 5). According to the method of Benedict *et al.* (1997), background fluorescence was subtracted from measured MFI and the parameters MFI_{max} and K_D were obtained from the fit to the hyperbolic equation $MFI = MFI_{max} \cdot [scFv]/(K_D + [scFv])$. This method allows the determination of the affinity directly on cells, which is more relevant for the intended application than the affinity to the isolated protein, which for this reason was not determined. From these data, K_D values of 76 nM (±18 nM) for binding of scFv 5R109 to rat CD86 and 12 μM (±4 μM) for binding of scFv 5R1 to rat CD86 were determined (Fig. 5).

To examine whether CD28–Fc, CTLA4–Fc and the selected scFv fragments compete with each other in binding to the surface-expressed CD86 protein, the reduction of fluorescence signal due to binding of CD28 or CTLA4 upon addition of scFv fragments was analyzed. When competing the binding of 100 nM CTLA4–Fc to CD86-expressing P815 cells with 3.3 μM scFv fragments, only the addition of scFv 5R109 succeeded in significantly reducing CTLA4–Fc binding by up to 90%. Similarly, in experiments with 3.3 μM scFv fragments competing with 25 nM CD28–Fc, only 5R109 showed a signal reduction. However, concentrations of monomeric scFv fragments used for these experiments were up to 130 times higher than those of dimeric CD28–Fc or CTLA4–Fc competitors (Fig. 4B and C).

Co-stimulation assay

Having shown that scFv 5R109 inhibits binding of soluble human CD28–Fc and murine CTLA4–Fc to rat CD86 expressed on the P815 cell surface, we analyzed whether

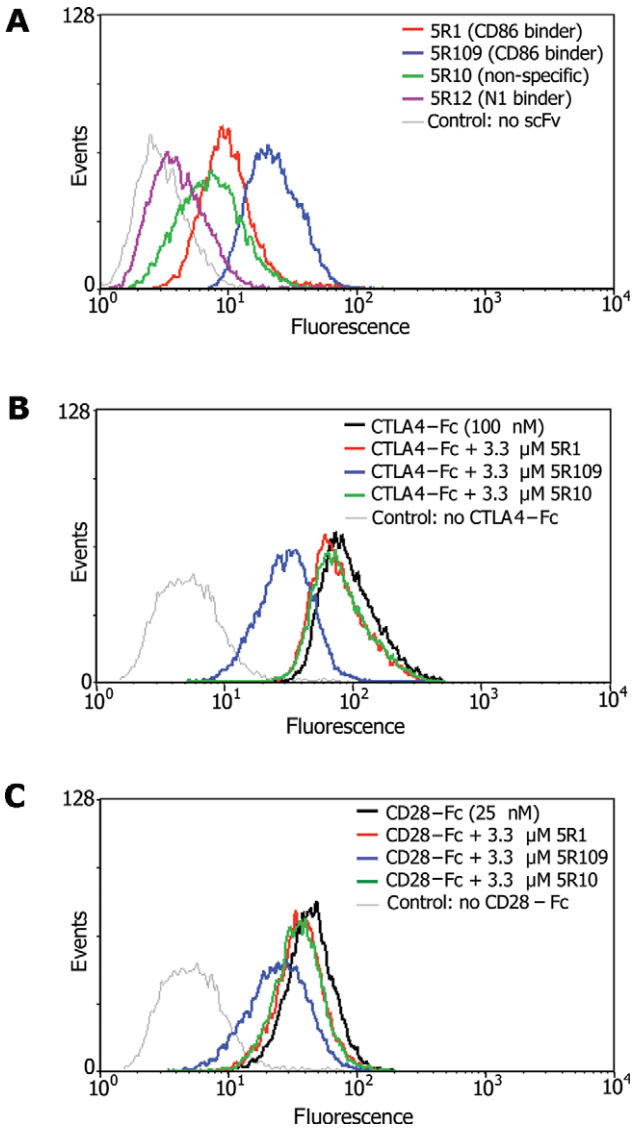

Fig. 4. Flow cytometry measurements. **(A)** Binding of selected scFv fragments to P815 cells expressing rat CD86. At identical concentrations (3.3 μM), scFv fragments bind with different strength to rat CD86 expressing cells. **(B)** Inhibition of CTLA4–Fc binding to CD86 expressing cells by scFv 5R109 only. **(C)** Inhibition of CD28–Fc binding to CD86 expressing cells by scFv 5R109 only. Grey line: no scFv or CD28 or CTLA4 added.

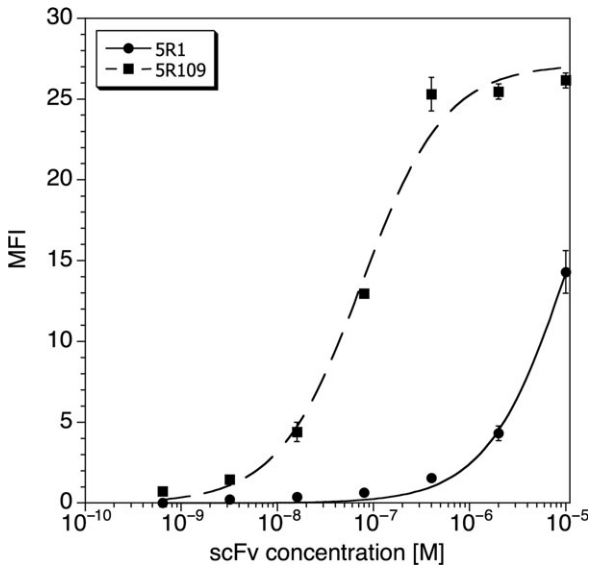

Fig. 5. Affinity determination of scFvs 5R1 and 5R109 to rat CD86 by flow cytometry measurements on whole cells. Parameters of a hyperbolic equation were fitted to MFI values obtained at different scFv fragment concentrations binding to CD86-expressing P815 cells. K_D values of 76 nM (±18 nM) and 12 μM (±4 μM) were obtained for scFvs 5R109 and 5R1, respectively, binding to rat CD86-expressing P815 cells. Data are presented as mean ± SE of two replicates.

scFv 5R109 also inhibits the CD86–CD28 interaction on cell surfaces leading to T-cell co-stimulation. We investigated in co-stimulation assays to what degree the addition of the selected antibody fragments decreases T-cell activation by reducing the co-stimulatory signal between rat CD80- or rat CD86-expressing mammalian cells and rat T-cells.

For this purpose, mouse P815 mastocytoma cells, expressing either rat CD86 or rat CD80 or no foreign protein on their surface, were used to provide the co-stimulatory signal. These mammalian cells were treated with mitomycin C to stop proliferation and co-cultured with rat T-cells from lymph nodes, in medium containing the agonistic anti-CD3 antibody G4.18 (Nicolls *et al.*, 1993). This antibody is a surrogate for TCR activation by the peptide–MHC complex.

In cultures with untransduced P815 cells no proliferation was found, but in cultures with CD80 or CD86 expressing cells, a strong thymidine incorporation was found (Fig. 6), which demonstrates the dependence of the CD3-induced T-cell activation on ligation of CD28 of the rat T-cells by co-stimulatory CD80 or CD86 molecules expressed by the transduced P815 cells. Addition of CTLA4–Fc, which blocks CD28 interaction with both CD80 and CD86, abolishes proliferation nearly completely, whereas the unspecific scFv 5R12 showed no effects. The CD86-specific scFv (5R109) inhibited only CD86- but not CD80-dependent proliferation (Fig. 6). Addition of scFv fragments to T-cells lacking co-stimulatory signal induction by CD80 or CD86 did not increase T-cell proliferation, excluding unspecific stimulatory effects by the scFv preparation.

Discussion

We have generated scFv fragments that inhibit the interaction of rat CD86 with T-cells. As it is essential to be able to make predictive experiments in an autologous rat model system, we had to devise reagents for this system. We could produce the IgV-like domain of rat CD86 in *E. coli* by refolding with a protocol previously shown to be successful for antibody scFv fragments. CD86 is a monomer consisting of two Ig-like domains (V-type and C-type) that has several potential glycosylation sites, two of which are in the N-terminal region of the V-type domain. As shown previously, the non-glycosylated IgV-like domain of CD86 has significant binding activity to CTLA4 (Zhang *et al.*, 2002). This is consistent with crystallographic studies, which localized the CTLA4 binding site in CD86 to residues in the GFCC′C″ surface, distant from the potential *N*-glycosylation sites (Schwartz *et al.*, 2001).

At the time the phage selections were carried out, no soluble rat CD86 had been commercially available, and therefore the antigen for selection and binder characterization had to be produced in our own laboratory. However, this allowed us to use as a selection target only the IgV-like domain of CD86, which interacts with CD28 and CTLA4. Two antibody fragments specifically binding to the refolded rat CD86 protein were obtained. To achieve higher binding affinities, an evolutionary approach to affinity maturation (Luginbühl *et al.*, 2006) of the obtained scFvs can be carried out. However, since an evaluation of such affinity-matured fragments can only be performed in conjunction with *in vivo* studies, this has not yet been undertaken.

As we were interested in the inhibitory properties of the scFv specific for rat CD86 in the context of T-cell co-stimulation, we carried out functional assays with CD86 protein on the surface of P815 mastocytoma cells. The selected scFv fragments indeed showed comparable binding properties to rat CD86 expressed as Fc-fusion proteins in mouse myeloma cells (Fig. 3) or to murine cells expressing rat CD86 on the surface (Fig. 4).

In inhibition assays performed with soluble mouse CTLA4–Fc and human CD28–Fc fusion proteins, the scFv fragment 5R109 did inhibit the binding of mouse CTLA4–Fc and of human CD28–Fc to rat CD86-expressing P815 cells by up to 90%. However, concentrations up to 130 times higher of the monovalent scFv had to be used than of the dimeric CD28–Fc or CTLA4–Fc constructs.

In subsequent co-stimulation assays, one of the scFv fragments was able to reduce T-cell proliferation by over 98%. As T-cell proliferation in co-stimulation assays as well as in the intact immune response depends on activation of the cells with a threshold characteristic, it still remains to be shown whether the inhibitory function observed in the cell culture assay will also be observed in transplantation experiments. Proteins inhibiting the interaction of CD80 and CD86 with CD28 have shown promising results in the prevention of graft rejection. Monoclonal antibodies against CD80 or CD86 have been used systemically in tissue transplantation experiments (Kagaya *et al.*, 2002; Montgomery *et al.*, 2002). A soluble CTLA4–Fc fusion protein developed by Bristol-Myers Squibb has been approved by the FDA for use in rheumatoid arthritis (Genant, 2005; Genovese *et al.*, 2005). Monoclonal antibodies targeted against CD28 have also been shown to delay allograft rejection in rat models (Dong *et al.*, 2002; Otto *et al.*, 2002).

The application of such inhibitors in preventing graft rejection in corneal transplantation would be very attractive, as the protein can be applied topically at very high concentrations. For such an approach, it is required, however, that the protein is small enough to diffuse efficiently through the tissue. Therefore, IgG molecules would not be suitable for topical application in corneal transplantation, as they are too large to diffuse through the cornea. Similarly, CTLA4–Fc fusions would show the same limitation. By contrast, monovalent scFv fragments are smaller than this diffusion barrier. This monovalence bears the intrinsic disadvantage that avidity cannot be exploited. However, monovalence is not only dictated by diffusion: it is also essential that the binding molecule should only reduce the availability of the target to its binding partners without cross-linking the target molecules and thereby potentially induce a cellular signal. Therefore, bivalent diabodies would also potentially run the danger of triggering an activation.

Different libraries have been used previously in selection for binding to the molecules of the second signal. Starting with an antibody fragment library consisting of V_L domains only, van den Beucken *et al.* (2001) have selected molecules binding to human CD80 and CD86, which, after affinity maturation, had a K_D of 191 nM to CD80. Due to their very small size (15 kDa), these proteins could also be of interest for clinical applications. Using a similar small domain of protein A as a scaffold, Sandström *et al.* (2003) have selected proteins binding to human CD28 by phage display, however, with rather weak affinity (K_D of 8.5 μM). It is, at present,

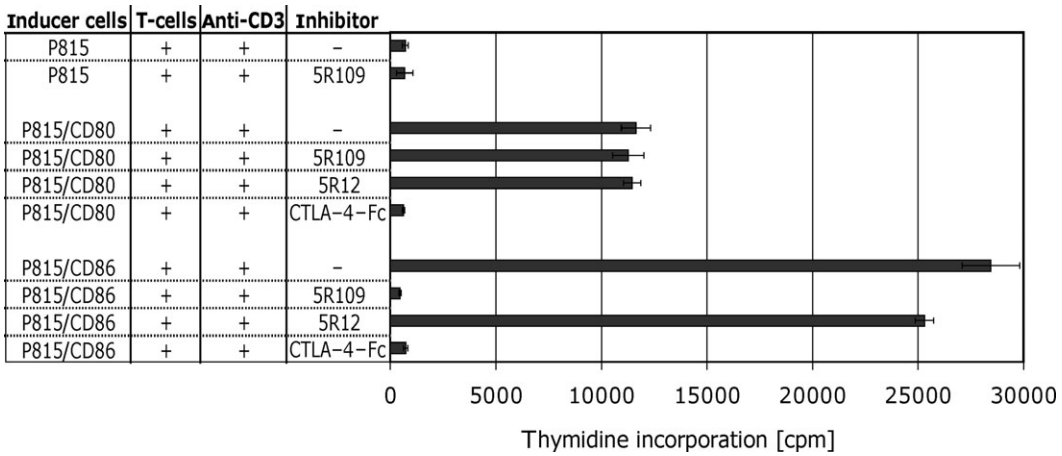

Fig. 6. Co-stimulation assay measuring [^{3}H]-thymidine incorporation upon T-cell proliferation. Rat T-cells activated by agonistic anti-CD3 antibody G4.18 proliferate in co-culture with rat CD80- or rat CD86-expressing P815 cells. Different CD80- or CD86-binding proteins inhibit this proliferation. Data are presented as mean ± SE of three replicates. (P815: non-expressing cell line; P815/CD80: cell line expressing rat CD80; P815/CD86: cell line expressing rat CD86.)

unclear which molecular format would be most suited for the topical applications as envisioned here. Beside size and affinity, the protein should not, of course, be immunogenic by itself especially in such an application, and this is a potential issue with all scaffolds alike (Binz *et al.*, 2005).

The rat CD86-binding scFv fragment 5R109 obtained here represents a very interesting lead molecule for future cornea transplantation experiments, even though it is probably in need of additional affinity maturation (Luginbühl *et al.*, 2006). As the CD86–CD28 interaction is important in the initiation of the immune response, blocking this interaction is expected to suppress the immune reaction. However, if the blockage by the described CD86-binding scFv fragments should be not sufficient for suppression, combined administration of CD80- or CD28-binding scFv fragments is expected to further increase this effect. Because of the high structural similarity between CD28, CD80 and CD86, the selection strategy described here can probably be applied to these target proteins as well.

Due to the lack of cross-species binding as observed in ELISA assays, the scFv antibody fragment 5R109 is not expected to be effective in other species than rat. However, it may be more important to do meaningful proof-of-principle experiments with a system directly adapted to the animal model than to accept any compromise in affinity or exact binding mode, only to generate cross-species reagents. To obtain functionality in mouse or human systems, the scFv fragment 5R109 would have to be modified by rational design and/or directed evolution or a new molecule would have to be selected binding to the corresponding epitope on human CD86. Nevertheless, experiments in the rat animal model with such selected antibody fragments will give valuable information about the efficiency of anti-CD86 antibody fragments for immuno-modulation in cornea transplantation.

Acknowledgments

We thank Patricia Martin Killias for her help in flow cytometry and Stefan Wiehr for his help in co-stimulation experiments. This project has been funded by the Swiss Science Foundation (Grant SNF #4046-058715).

References

Benedict,C.A., MacKrell,A.J. and Anderson,W.F. (1997) *J. Immunol. Methods*, **201**, 223–231.
Binz,H.K., Amstutz,P., Kohl,A., Stumpp,M.T., Briand,C., Forrer,P., Grütter,M.G. and Plückthun,A. (2004) *Nat. Biotechnol.*, **22**, 575–582.
Binz,H.K., Amstutz,P. and Plückthun,A. (2005) *Nat. Biotechnol.*, **23**, 1257–1268.
Bonnevier,J.L., Yarke,C.A. and Mueller,D.L. (2006) *Eur. J. Immunol.*, **36**, 1583–1597.
Bugeon,L. and Dallman,M.J. (2000) *Am. J. Respir. Crit. Care Med.*, **162**, S164–S168.
Carreno,B.M. and Collins,M. (2002) *Annu. Rev. Immunol.*, **20**, 29–53.
Colombetti,S., Basso,V., Mueller,D.L. and Mondino,A. (2006) *J. Immunol.*, **176**, 2730–2738.
Coyle,A.J. and Gutierrez-Ramos,J.C. (2001) *Nat. Immunol.*, **2**, 203–209.
Cull,M.G. and Schatz,P.J. (2000) *Methods Enzymol.*, **326**, 430–440.
Dong,V.M., Yuan,X., Coito,A.J., Waaga,A.M., Sayegh,M.H. and Chandraker,A. (2002) *Transplantation*, **73**, 1310–1317.
Frisch,C., Brocks,B., Ostendorp,R., Hoess,A., von Rüden,T. and Kretzschmar,T. (2003) *J. Immunol. Methods*, **275**, 203–212.
Ge,L., Knappik,A., Pack,P., Freund,C. and Plückthun,A. (1995) In Borrebaeck,C.A.K. (eds.), *Antibody Engineering*. Oxford University Press, Inc., New York, pp. 229–266.
Gebhardt,B.M., Hodkin,M., Varnell,E.D. and Kaufman,H.E. (1999) *Cornea*, **18**, 314–320.
Genant,H., Peterfy,C., Paira,S., Gomez-Reino,J., Miaux,Y., Wu,C., Jiang,Y., Sieffert,M., Ge,Z. and Aranda,R., *et al*. (2005) *Ann. Rheum. Dis.*, **64** (Suppl 3), 175.
Genovese,M.C., Becker,J.C., Schiff,M., Luggen,M., Sherrer,Y., Kremer,J., Birbara,C., Box,J., Natarajan,K. and Nuamah,I., *et al*. (2005) *N. Engl. J. Med.*, **353**, 1114–1123
Hoffmann,F., Zhang,E.P., Pohl,T., Kunzendorf,U., Wachtlin,J. and Bulfone-Paus,S. (1997) *Graefes Arch. Clin. Exp. Ophthalmol.*, **235**, 535–540.
Kagaya,F., Hori,J., Kamiya,K., Kaji,Y., Oshika,T., Amano,S., Yamagami,S., Tsuru,T., Tanaka,S. and Matsuda,H., *et al*. (2002) *Exp. Eye Res.*, **74**, 131–139.
Knappik,A., Ge,L., Honegger,A., Pack,P., Fischer,M., Wellnhofer,G., Hoess,A., Wölle,J., Plückthun,A. and Virnekäs,B. (2000) *J. Mol. Biol.*, **296**, 57–86.
Leach,D.R., Krummel,M.F. and Allison,J.P. (1996) *Science*, **271**, 1734–1736.
Lenschow,D.J., Herold,K.C., Rhee,L., Patel,B., Koons,A., Qin,H.Y., Fuchs,E., Singh,B., Thompson,C.B. and Bluestone,J.A. (1996) *Immunity*, **5**, 285–293.
Liang,L. and Sha,W.C. (2002) *Curr. Opin. Immunol.*, **14**, 384–390.
Linsley,P.S. and Ledbetter,J.A. (1993) *Annu. Rev. Immunol.*, **11**, 191–212.
Lubkowski,J., Hennecke,F., Plückthun,A. and Wlodawer,A. (1998) *Nat. Struct. Biol.*, **5**, 140–147.

Luginbühl,B., Kanyo,Z., Jones,R.M., Fletterick,R.J., Prusiner,S.B., Cohen,F.E., Williamson,R.A., Burton,D.R. and Plückthun,A. (2006) *J. Mol. Biol.*, **363**, 75–97.
Maeda,K., Sato,T., Azuma,M., Yagita,H. and Okumura,K. (1997) *Int. Immunol.*, **9**, 993–1000.
Montgomery,S.P., Xu,H., Tadaki,D.K., Celniker,A., Burkly,L.C., Berning,J.D., Cruzata,F., Elster,E.A., Gray,G. and Kampen,R.L., *et al*. (2002) *Transplantation*, **74**, 1365–1369.
Nicolls,M.R., Aversa,G.G., Pearce,N.W., Spinelli,A., Berger,M.F., Gurley,K.E. and Hall,B.M. (1993) *Transplantation*, **55**, 459–468.
Otto,C., Feuerlein,S., Timmermann,W., Ulrichs,K., Hünig,T., Thiede,A. and Gassel,H. (2002) *Transplant. Proc.*, **34**, 2376.
Peach,R.J., Bajorath,J., Naemura,J., Leytze,G., Greene,J., Aruffo,A. and Linsley,P.S. (1995) *J. Biol. Chem.*, **270**, 21181–21187.
Proba,K., Honegger,A. and Plückthun,A. (1997) *J. Mol. Biol.*, **265**, 161–172.
Rennert,P., Furlong,K., Jellis,C., Greenfield,E., Freeman,G.J., Ueda,Y., Levine,B., June,C.H. and Gray,G.S. (1997) *Int. Immunol.*, **9**, 805–813.
Rogers,N.J., Game,D.S., Camara,N.O., Jackson,I.M., Lombardi,G. and Lechler,R.I. (2005) *Eur. J. Immunol.*, **35**, 2909–2919.
Saito,T. (1998) *Curr. Opin. Immunol.*, **10**, 313–321.
Sandström,K., Xu,Z., Forsberg,G. and Nygren,P.Å. (2003) *Protein Eng.*, **16**, 691–697.
Schwartz,J.C., Zhang,X., Fedorov,A.A., Nathenson,S.G. and Almo,S.C. (2001) *Nature*, **410**, 604–608.
Shapiro,V.S., Truitt,K.E., Imboden,J.B. and Weiss,A. (1997) *Mol. Cell. Biol.*, **17**, 4051–4058.
Stüber,D., Matile,H. and Garotta,G. (1990) In Lefkovits,I. and Pernis,B. (eds.), *Immunological Methods*. Academic Press, Orlando, FL, USA, 121–152.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
Thiel,M.A., Coster,D.J., Standfield,S.D., Brereton,H.M., Mavrangelos,C., Zola,H., Taylor,S., Yusim,A. and Williams,K.A. (2002) *Clin. Exp. Immunol.*, **128**, 67–74.
Thiel,M.A., Steiger,J.U., O'Connell,P.J., Lehnert,A.M., Coster,D.J. and Williams,K.A. (2005) *Clin. Experiment. Ophthalmol.*, **33**, 176–180.
van den Beucken,T., van Neer,N., Sablon,E., Desmet,J., Celis,L., Hoogenboom,H.R. and Hufton,S.E. (2001) *J. Mol. Biol.*, **310**, 591–601.
Zhang,X., Schwartz,J.C., Almo,S.C. and Nathenson,S.G. (2002) *Protein Expr. Purif.*, **25**, 105–113.

Received December 01, 2006; accepted December 06, 2006

Edited by Paul Carter