Construction and expression in the yeast *Pichia pastoris* of functionally active soluble forms of the human costimulatory molecules B7-1 and B7-2 and the B7 counter-receptor CTLA-4

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Abstract We have generated soluble recombinant forms of the costimulatory molecules B7-1 and B7-2, and their counter-receptor CTLA-4 using a yeast *Pichia pastoris* expression system. Fragments comprising the extracellular domains of human B7-1, B7-2, and CTLA-4 molecules were expressed at high levels and could be purified from culture supernatants following a simple one-step purification protocol. The recombinant proteins retained their functionality and specific binding to their natural counterparts could be demonstrated by FACS analysis. In T cell proliferation assays costimulatory activity of immobilized B7-1 and B7-2 proteins in the presence of an anti-CD3 antibody was observed with the B7-1 protein being more potent than B7-2.

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Key words: *Pichia pastoris*; Heterologous expression; Human B7; Human CTLA-4; T cell costimulation

1. Introduction

For activation and clonal expansion T cells require costimulatory signals in addition to the primary signal provided by the T cell receptor (TCR), which interacts with antigenic peptides in the context of major histocompatibility complex class I or II molecules and confers antigen specificity [1]. The second, costimulatory signal can be provided by cell surface molecules on antigen presenting cells such as B7-1 (CD80) and B7-2 (CD86) which interact with their counter-receptors on T cells [2].

CD28 is the major costimulatory signal receptor for $CD4^+$ and $CD8^+$ T cells and members of the B7 family are its natural ligands. TCR and costimulatory signals together induce T cell proliferation and the production of cytokines. CTLA-4 binds to members of the B7 family with approximately 20-fold higher affinity than CD28, but in contrast to CD28 transmits a negative signal to the T cell which contributes to the termination of the T cell response [3,4].

B7-1 and B7-2 as well as their counter-receptors CD28 and CTLA-4 are transmembrane glycoproteins of the Ig-superfamily [5]. B7-1 and B7-2 are expressed on the cell surface as monomeric proteins and carry both an IgC and an IgV loop in their extracellular domains [6,7]. Monomeric and disulfide-linked homodimeric forms of CD28 and CTLA-4 proteins which both contain a single extracellular IgV domain have been described [8,9]. Soluble fusion proteins consisting of the extracellular domains of B7-1, B7-2, or CTLA-4 genetically fused to an IgG Fc region have been produced in mammalian cells as tools to study the interaction of costimulatory ligands and receptors, and as reagents of potential therapeutic value [10]. CTLA-4-Ig fusion protein is a potent inhibitor of T cell activation in vitro and in experimental model systems in vivo has been shown to prevent autoimmune disease and to suppress tissue graft rejection in transplantation experiments [11–13].

So far only few attempts have been made to utilize non-mammalian expression systems for the production of lymphocyte cell surface antigens [14–17]. Since the majority of lymphocyte surface antigens are post-translationally modified which might be crucial for their biological activity, expression in *Escherichia coli* has its limitations. In contrast, despite differences in the glycosylation machinery of the yeast *Pichia pastoris* and mammalian cells, several heterologous glycoproteins have been reported to be readily expressed, correctly folded, and glycosylated in *P. pastoris* yielding biologically active proteins [18].

Here we have extended the use of the *P. pastoris* expression system to the production of the costimulatory B7-1 and B7-2 molecules and their counter-receptor CTLA-4. The extracellular domains of human B7-1, B7-2, and CTLA-4 were expressed at high levels as soluble recombinant proteins and purified from culture supernatants following a simple one-step purification protocol. The yeast-produced molecules retained their functionality in a variety of in vitro assays suggesting *P. pastoris* as a useful alternative to mammalian systems for the expression of soluble forms of lymphocyte cell surface molecules.

2. Materials and methods

2.1. Cell lines and cell culture conditions

CHO and CHO-CTLA-4 cells (see below) were maintained in MEMα with deoxyribonucleosides (Gibco BRL), containing 2 mM glutamine, 50 μM β-mercaptoethanol, 10% heat-inactivated fetal bovine serum (FBS), and 1 mg/ml G418 (CHO-CTLA-4). The acute monocytic leukemia cell line THP-1 (ATCC) and B7-1-transfected THP-1 cells (kindly provided by A. Lanzavecchia, Basel) were grown in RPMI medium supplemented with 2 mM glutamine, 5 mM Na-pyruvate, 5 mM non-essential amino acids, 50 μM β-mercaptoethanol, 10% heat-inactivated FCS, and 1 mg/ml G418 (THP-1-B7-1). The *P. pastoris* GS 115 yeast cells (Invitrogen, Leek, Netherlands) were propagated in buffered minimal glycerol-complex medium (BMGY) and expression of recombinant proteins was induced in buffered minimal methanol-complex medium (BMMY) according to the distributor's recommendations.

2.2. Construction of plasmids for the expression of soluble extracellular domain of human B7-1 and B7-2

A cDNA fragment encoding the extracellular domain of human B7-

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1 (amino acid residues 1–208; referred to as B7-1_{208}) was derived by PCR using full-length B7-1 cDNA (kindly provided by U. Zangemeister-Wittke, Zurich) as a template and the oligonucleotides B7-1-sense 5′-TATGTCGACGCTAGCGTTATCCACGTGACC-3′ and B7-1-antisense 5′-TATCTCTAGACCATCGATGTTATCAGGAAAATG-CTCTTGC-3′ which introduce *Sal*I and *Nhe*I restriction sites at the 5′-end and *Cla*I and *Xba*I restriction sites at the 3′-end of the PCR product. Likewise a cDNA fragment encoding the extracellular domain of human B7-2 (amino acid residues 1–225; referred to as B7-2_{225}) was derived by reverse transcription of RNA from human peripheral blood mononuclear cells (PBMC) followed by PCR. cDNA encoding amino acids 1–225 of human B7-2 was amplified using the oligonucleotides B7-2-sense 5′-AAAAGTCGACGCTAGCGCTGCT-CCTCTG-3′ and B7-2-antisense 5′-AAAACTCTAGAGATCTATC-GATAGGAATGTGGTCTGG-3′ which introduce *Sal*I and *Nhe*I restriction sites at the 5′-end and *Cla*I, *Bgl*II, and *Xba*I restriction sites at the 3′-end of the PCR product.

The amplified B7-1_{208} and B7-2_{225} cDNA fragments were ligated in frame 5′ to a synthetic sequence which encodes the Myc tag recognized by the monoclonal antibody (Mab) 9E10 [19] and a cluster of 6 histidine residues (His tag) in a modified pBluescript KS$^+$ (Stratagene, Heidelberg, Germany). For expression in the yeast *P. pastoris* the B7-1_{208} and B7-2_{225} encoding fragments including Myc and His tags were isolated from the cloning vector and inserted into the yeast expression vector pPIC9 (Invitrogen) resulting in the plasmids pPIC9-B7-1_{208} and pPIC9-B7-2_{225}.

2.3. Construction of plasmids for the expression of the extracellular domain of human CTLA-4

A cDNA fragment encoding the extracellular domain of human CTLA-4 (amino acid residues 1–125; referred to as CTLA-4_{125}) was derived by PCR using CTLA-4-Ig cDNA (kindly provided by K. Pfeffer, Munich) as a template and the oligonucleotides CTLA-4-sense 5′-TTTGTCGACGCTAGCGCAATGCACGTGGCC-3′ and CTLA-4-antisense 5′-TTTTCTAGACCATCCGAATCTGGG-3′ which introduce *Sal*I and *Nhe*I restriction sites at the 5′-end and an *Xba*I restriction site at the 3′-end of the PCR product. The amplified CTLA-4_{125} cDNA fragment was fused to Myc and His tags and subcloned into the *P. pastoris* expression vector pPIC9 as described above resulting in the construct pPIC9-CTLA-4_{125}. CHO cells expressing the GPI-linked extracellular domain of human CTLA-4 on their surface were generated by introducing via PCR *Hin*dIII and *Spe*I restriction sites at the 5′- and 3′-ends of the respective CTLA-4 cDNA fragment (kindly provided by P. Dellabona and G. Casorati, Milan, Italy). The PCR product was ligated 5′ to a DNA fragment encoding the GPI linker (123 bp fragment) of the human urokinase receptor [20] in the plasmid pRC/RSV. CHO cells were transfected with the construct using liposomal transfection (DOTAP, Boehringer Mannheim, Mannheim, Germany), stable transfectants were selected with G418 and tested for cell surface CTLA-4 expression with B7-1-Ig (kindly provided by P. Dellabona and G. Casorati).

2.4. Expression in yeast and purification of recombinant proteins

Derivatives of the pPIC9 expression vector were linearized by *Sal*I digestion and used for transformation of *P. pastoris* GS115 cells by electroporation [21]. His4$^+$/methanol-utilization$^+$ (mut$^+$) yeast colonies were isolated on selection media following established protocols [22] and the phenotype of the resulting clones was confirmed by PCR using AOX1 5′- and 3′-primers (Invitrogen).

For expression of recombinant proteins single yeast colonies were grown to an OD_{600} of 3 in BMGY medium, pH 7.5, the medium was exchanged with methanol-containing BMMY medium, pH 7.5, and protein expression was induced for 72 h at 30°C. Yeast cells were removed by centrifugation at 20000×*g*. Supernatants containing soluble B7 and CTLA-4 proteins were passed through a 45 μm filter, applied onto a Ni^{2+}-saturated chelating sepharose column (Pharmacia Biotech, Freiburg, Germany) and the recombinant proteins specifically bound via their C-terminal polyhistidine tag were eluted with 250 mM imidazole in PBS. Fractions containing the fusion protein were identified by SDS-PAGE and immunoblotting with Mab 9E10, pooled, concentrated by ultrafiltration, and dialyzed against PBS. Post-translational modification of recombinant proteins was analyzed in a deglycosylation reaction. 0.2 μg each of purified proteins were heated to 100°C for 10 min in PBS containing 0.1% SDS. Triton X-100 was added to a final concentration of 1% and the proteins were incubated with 1 U of *N*-glycosidase F (Boehringer Mannheim) for 16 h at 37°C in a total reaction volume of 100 μl.

2.5. Binding assays

The binding of soluble B7-1_{208} and B7-2_{225} to the B7 counter-receptor CTLA-4 was determined by FACS analysis using CHO-CTLA-4 cells. A single cell suspension was prepared by treatment with trypsin, and 5×10^5 CHO-CTLA-4 cells were incubated for 45 min at 4°C with 0.2–1 μg each of B7-2_{225} or B7-1_{208} protein, followed by incubation with 3 μg of Mab 9E10 and phycoerythrin-labeled goat anti-mouse IgG (Rockland, Gilbertsville, PA) for 30 min. Binding of B7 proteins was detected using a FACScan (Becton-Dickinson, Heidelberg, Germany). CHO cells served as a control. Similarly, the binding of soluble CTLA-4_{125} to B7-1 was determined by FACS analysis using B7-1 expressing THP-B7-1 cells, and parental B7-1-negative THP-1 cells as a control.

2.6. T cell proliferation assays

Primary T cells were derived from the spleens of BALB/c mice exactly as described [23]. Flat bottom 96 well plates were coated overnight with the mitogenic anti-mouse CD3 Mab 2C11 [24] (kindly provided by S. Desrivieres, Freiburg) alone or in combination with purified soluble B7-1_{208} or B7-2_{225}. The plates were washed with PBS and 1×10^5 splenic T cells in RPMI medium supplemented with 8% FCS were added to each well. After incubation at 37°C for 60 h the cells were pulsed with 0.25 μCi/well [^{3}H]thymidine (DuPont, Boston, MA) for 12 h, the cultures were harvested, and the incorporation of [^{3}H]thymidine was measured with a liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

3. Results

3.1. Construction of plasmids for the expression in P. pastoris of the human costimulatory molecules B7-1 and B7-2, and the B7 counter receptor CTLA-4

cDNAs encoding the extracellular domains of the human costimulatory molecules B7-1_{208}, B7-2_{225}, and the B7 counter-receptor CTLA-4_{125} were derived by polymerase chain reaction with specific oligonucleotide primers. The respective cDNA fragments together with synthetic sequence tags at their 3′-ends were inserted into the yeast expression vector pPIC9. As shown in Fig. 1 the resulting plasmids pPIC9-B7-1_{208}, pPIC9-B7-2_{225}, and pPIC9-CTLA-4_{125} encode under the control of the methanol inducible alcohol oxidase 1 (AOX1) promoter fusion proteins consisting of an N-terminal α-factor secretion signal from yeast, the extracellular domain of the respective human protein, and C-terminal Myc and polyhistidine tags facilitating immunological detection with the monoclonal antibody (Mab) 9E10 [19] and purification via Ni^{2+} affinity chromatography, respectively.

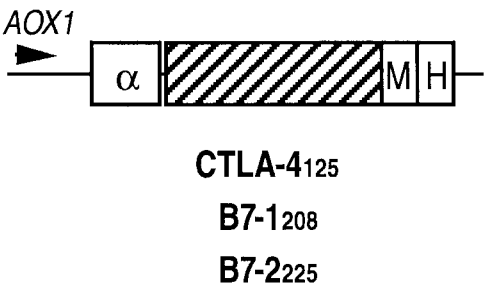


Fig. 1. Schematic representation of the expression cassettes in the yeast pPIC9 vector encoding CTLA-4_{125}, B7-1_{208}, or B7-2_{225}. Expression in the yeast *P. pastoris* is regulated by the alcohol oxidase promoter (AOX1) and is directed to the extracellular space via the yeast α-factor secretion signal (α). M, Myc tag; H, polyhistidine tag.

3.2. Expression and purification of CTLA-4_{125}, B7-1_{208} and B7-2_{225}

P. pastoris GS115 cells which carry the pPIC9 expression constructs stably integrated into the genome were obtained by electroporation of competent cells with plasmids linearized by *Sal*I digestion. Upon integration of the pPIC9 plasmids positive clones were identified by selection on minimal dextrose agar plates lacking histidine. The 5′- and 3′-sequences of the alcohol oxidase gene in the vector allow for integration into, and disruption of the genomic AOX1 locus, resulting in cells which grow very slowly in medium containing methanol as the only carbon source (mut^s). Due to the presence of the intact HIS4 gene in pPIC9 vectors, the expression cassettes can alternatively be integrated into the genomic HIS4 locus. The resulting cells grow normally with methanol as the only carbon source (mut$^+$). The phenotype of clones was characterized by PCR using genomic DNA as a template and 5′- and 3′-AOX1 primers. Expression of recombinant proteins by mut$^+$ clones was confirmed by immunoblot analysis of culture supernatants with Mab 9E10 (data not shown).

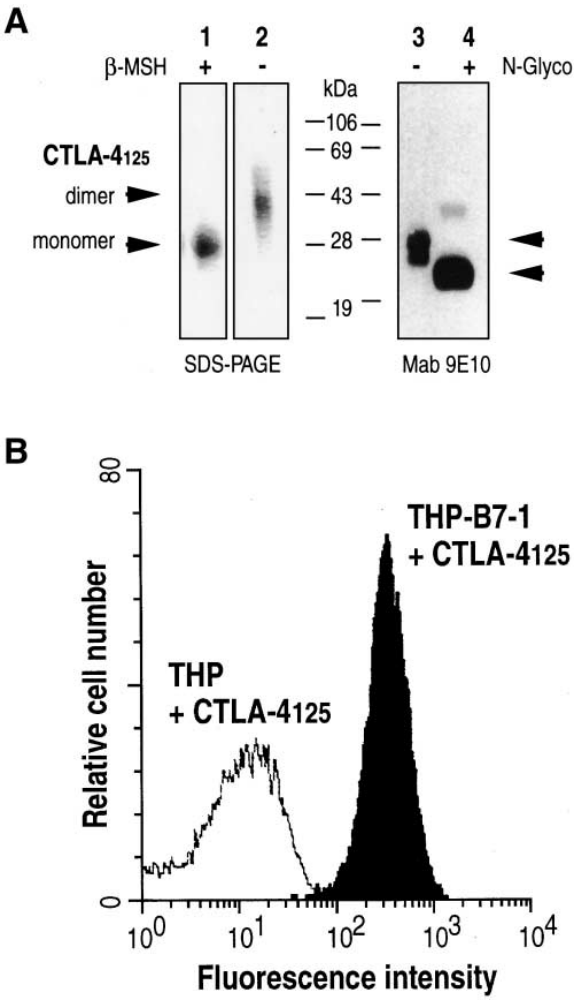


Fig. 2. A: SDS-PAGE analysis of purified CTLA-4_{125} in the presence (lane 1) or absence (lane 2) of the reducing agent β-mercaptoethanol (β-MSH). The positions of monomeric and dimeric forms of the protein are indicated. Lanes 3 and 4, monoclonal antibody 9E10 immunoblot analysis of purified CTLA-4_{125} before (lane 3) or after (lane 4) treatment with protein *N*-glycosidase F (N-Glyco). The positions of differently glycosylated forms of CTLA-4_{125} and of molecular weight standards are indicated. B: The binding of purified CTLA-4_{125} to THP-B7-1 cells and B7-negative THP control cells was detected by FACS analysis with monoclonal antibody 9E10 and phycoerythrin-labeled goat anti-mouse IgG.

Upon induction of expression in methanol-containing medium, CTLA-4_{125}, B7-1_{208} and B7-2_{225} proteins were efficiently secreted into the culture supernatant. Yeast cells were removed by centrifugation and the recombinant proteins were purified via Ni^{2+} affinity chromatography. SDS-PAGE analysis of the proteins revealed a purity of greater than 80% after a single round of purification (see below). The yield of soluble recombinant B7-1_{208} and B7-2_{225} proteins purified from 1 l of yeast culture supernatant was typically 10 mg. The yield of soluble recombinant CTLA-4_{125} was even higher with approximately 15 mg from 1 l of yeast culture.

3.3. Soluble CTLA-4_{125} is expressed as a dimer and binds to B7 molecules

Full-length CTLA-4 exists mainly as a disulfide-linked homodimeric molecule on the surface of T cells. Likewise, yeast-expressed soluble CTLA-4_{125} was also found mainly in a dimeric disulfide-linked form as analyzed by SDS-PAGE under reducing and non-reducing conditions (Fig. 2A, lanes 1 and 2). In contrast to the calculated molecular weight of 17.1 kDa the protein migrates as a smear of bands with apparent molecular masses of approximately 26–32 kDa (monomeric CTLA-4_{125}) and 35–50 kDa (dimeric CTLA-4_{125}). *N*-Glycosidase F treatment of the protein reduced the apparent molecular mass indicating that the higher molecular mass of yeast-expressed CTLA-4_{125} protein is mainly due to post-translational modification by *N*-linked glycosylation (Fig. 2A, lanes 3 and 4).

To investigate the functionality of soluble CTLA-4_{125} the binding of the recombinant protein to B7-1 on the surface of cells was tested by FACS analysis. THP-B7-1 monocytoma cells stably transfected with B7-1 cDNA were incubated with CTLA-4_{125} and specifically bound protein was detected with Mab 9E10 and phycoerythrin-labeled goat anti-mouse IgG. The results are shown in Fig. 2B. Strong binding of CTLA-4_{125} to THP-B7-1 cells but not to B7-negative parental THP cells could be detected indicating that yeast-expressed soluble CTLA4 is functionally active and interacts with B7.

3.4. Characterization of soluble B7-1_{208} and B7-2_{225}

Soluble B7-1_{208} and B7-2_{225} proteins were produced as monomeric molecules as determined by SDS-PAGE under non-reducing conditions (data not shown). Like in the case of CTLA-4_{125} the apparent molecular weight of the yeast-expressed B7 proteins was higher than their calculated molecular weight. B7-1_{208} (calculated M_r of 28.5 kDa) and B7-2_{225} (calculated M_r of 30.2 kDa) migrate as several distinct bands or a smear of bands ranging from 60 to approximately 100 kDa (Fig. 3A,B, lane 1). *N*-Glycosidase F treatment reduced the apparent molecular mass of the proteins indicating that they are post-translationally modified by *N*-linked glycosylation (Fig. 3A,B, lanes 2 and 3).

The binding of soluble B7-1_{208} and B7-2_{225} proteins to the B7 counter receptor CTLA-4 was tested by FACS analysis. CHO-CTLA-4 cells expressing GPI-linked CTLA-4 on their surface were incubated with B7-1_{208} or B7-2_{225} and specifically bound protein was detected with Mab 9E10 and phycoerythrin-labeled goat anti-mouse IgG. The results are shown in

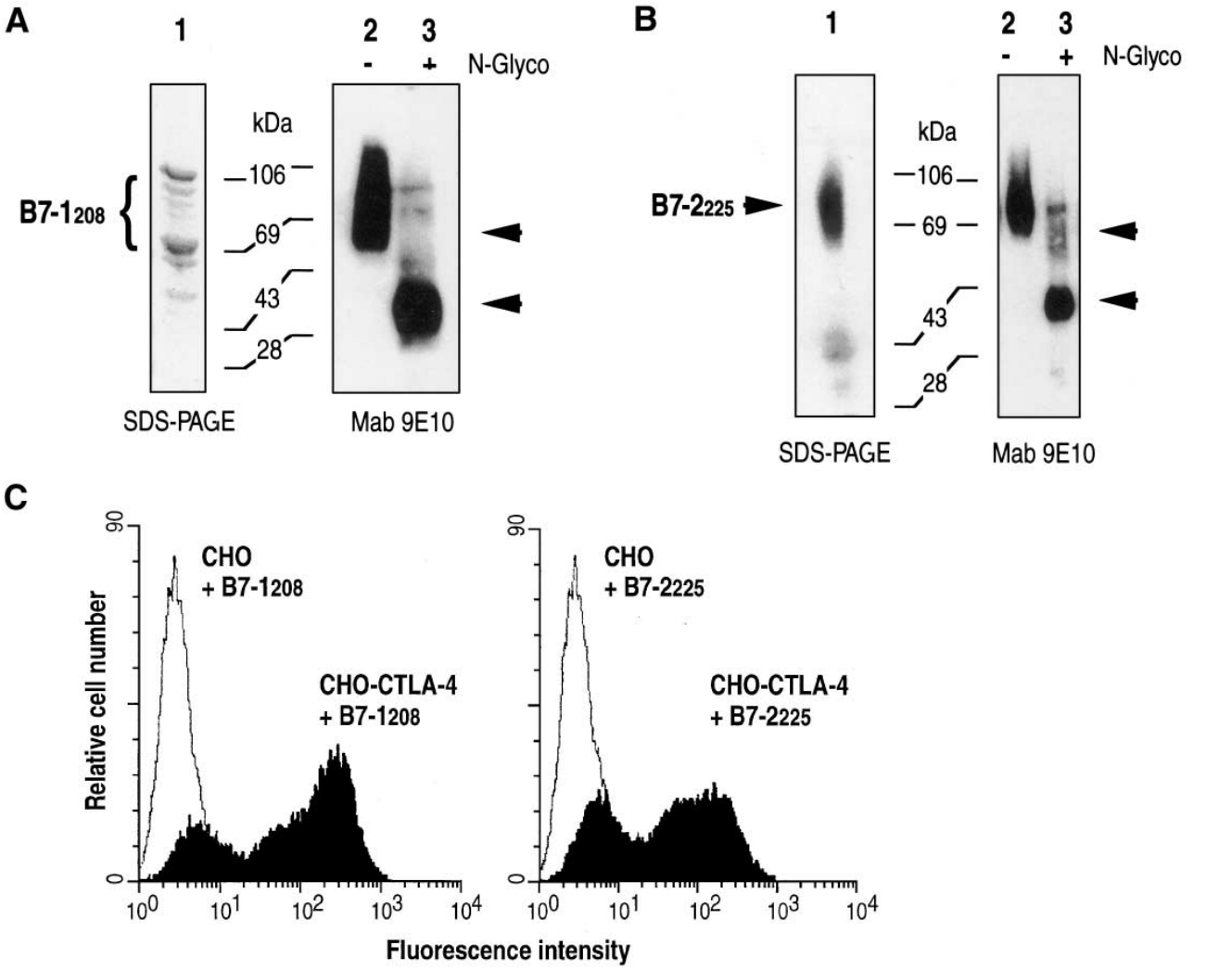


Fig. 3. SDS-PAGE analysis of purified B7-1_{208} (A) and B7-2_{225} (B) under reducing conditions (lanes 1). The positions of B7-1_{208} and B7-2_{225} are indicated. Lanes 2 and 3, monoclonal antibody 9E10 immunoblot analysis of purified B7 proteins before (lane 2) or after (lane 3) treatment with protein *N*-glycosidase F (N-Glyco). The positions of differently glycosylated forms of B7-1_{208} and B7-2_{225}, and of molecular weight standards are indicated. C: The binding of B7-1_{208} and B7-2_{225} to CHO-CTLA-4 cells or CTLA-4-negative CHO control cells was detected by FACS analysis with monoclonal antibody 9E10 and phycoerythrin-labeled goat anti-mouse IgG.

Fig. 3C. Binding of both proteins, B7-1_{208} (left panel) and B7-2_{225} (right panel) to CHO-CTLA-4 cells but not to CHO control cells could be detected. Incubation of CHO-CTLA-4 cells with soluble B7 proteins in the presence of a 50-fold molar excess of soluble CTLA-4_{125} blocked the binding of B7-1_{208} and B7-2_{225} which further confirmed the specificity of the interaction (data not shown). These results indicate that the yeast-expressed B7 proteins are functionally active and interact with B7 counter receptors.

3.5. B7-1_{208} and B7-2_{225} enhance T cell proliferation

It has been shown previously that a recombinant B7-1-Ig fusion protein enhanced T cell proliferation in combination with a mitogenic anti-CD3 antibody when it was immobilized on a plastic surface, but not as a soluble molecule [6]. In order to characterize the costimulatory activity of the B7-1_{208} and B7-2_{225} proteins, similar experiments were performed. The wells of 96 well plates were coated overnight with 1 μg/ml of the mitogenic anti-CD3 Mab 2C11 [24] alone or in combination with increasing concentrations of 0.5–50 μg/ml of purified B7-1_{208}. T cells isolated from the spleens of BALB/c mice were added to the wells, and proliferation of the cells was measured via the incorporation of [^{3}H]thymidine. The results are shown in Fig. 4A. B7-1_{208} enhanced the proliferation of T cells in a dose-dependent manner. In a similar experiment using 1 μg/ml of anti-CD3 Mab and 0.5–50 μg/ml of purified B7-2_{225}, the B7-2 protein failed to provide a costimulatory signal for T cells (data not shown). However, when the anti-CD3 antibody was applied at a higher concentration (5 μg/ml) both recombinant proteins B7-1_{208} and B7-2_{225} were effective in enhancing T cell proliferation (Fig. 4B). In several independent experiments the costimulatory activity of immobilized B7-1_{208} was higher than that of B7-2_{225} resulting in a consistent 2–3-fold increase in T cell proliferation in comparison to cells treated only with anti-CD3 antibody. In contrast, B7-2_{225} led to a significant but only moderate enhancement of [^{3}H]thymidine incorporation in T cells co-treated with anti-CD3 Mab (Fig. 4B).

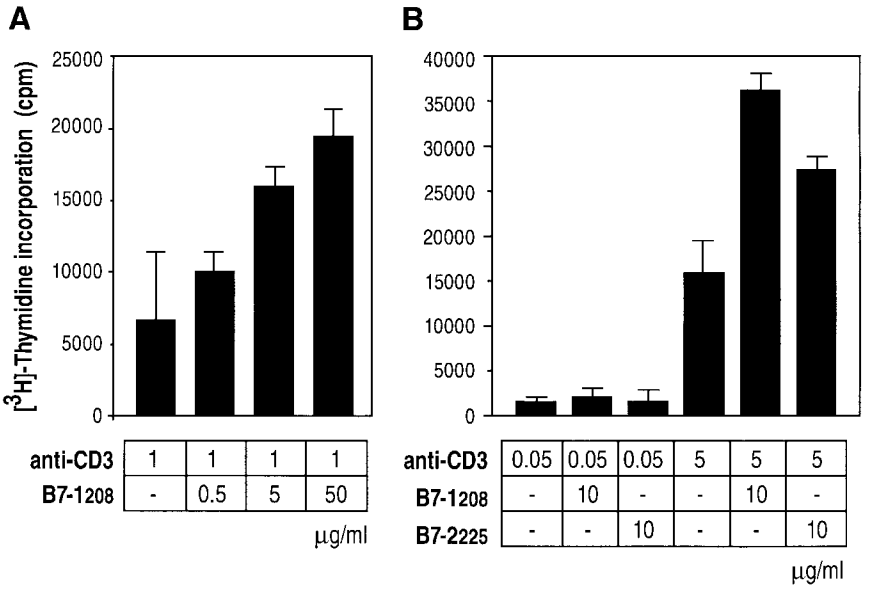


Fig. 4. Costimulation of primary T cells by B7-1_{208} and B7-2_{225}. Splenic T cells from BALB/c mice were incubated with the indicated concentrations of immobilized anti-CD3 monoclonal antibody and purified B7-1_{208} (A, B) or B7-2_{225} (B). Proliferation of cells was measured by [^{3}H]thymidine incorporation. Each value was determined in triplicate. The standard deviation is represented by error bars.

4. Discussion

Recombinant lymphocyte cell surface molecules have helped to study their biological function and to uncover their complex interactions with ligands and receptors. Nonetheless to date only few lymphocyte antigens have been produced in non-mammalian expression systems although the yield of recombinant protein from mammalian cells is usually low and protein purification is complicated.

The yeast *P. pastoris* has proved to be a valuable tool for the expression of a wide variety of heterologous proteins [18]. However, data on the expression of lymphocyte cell surface molecules in yeast are still very limited. We have developed a *P. pastoris* expression system for the production of soluble forms of human B7-1 and B7-2 and their counter-receptor CTLA-4. Recombinant proteins comprising the respective extracellular domains were expressed at high levels and could be purified from culture supernatants following a simple one-step purification protocol. The yeast-produced molecules retained their functionality and specific binding to their natural counterparts could be demonstrated by FACS analysis. In T cell proliferation assays costimulation of T cells by immobilized B7-1_{208} and B7-2_{225} in the presence of an anti-CD3 antibody was observed. In these experiments B7-1_{208} appeared to be superior in its activity to B7-2_{225}. It has been demonstrated that B7-1 binds to CD28 or CTLA-4 with different kinetics than B7-2 [25] which might explain why B7-2_{225} in comparison to B7-1_{208} is less efficient in enhancing T cell proliferation.

We have also produced recombinant B7-1_{208} and B7-2_{225} proteins in an *E. coli* expression system. However, in contrast to the molecules produced in yeast the bacterially expressed proteins failed to bind to the appropriate counter-receptor (data not shown). Although we cannot rule out that more sophisticated purification or refolding protocols would result in functionally active bacterial proteins, it is possible that glycosylation of B7-1 and B7-2 is a prerequisite for correct protein folding and efficient binding to their counter-receptors.

Endogenously expressed costimulatory molecules are highly glycosylated in their extracellular domains. Judged from the apparent molecular weight of CTLA-4_{125}, B7-1_{208}, and B7-2_{225} in SDS-PAGE the degree of glycosylation of *P. pastoris* expressed proteins and endogenously expressed mammalian costimulatory molecules appears to be similar [6–9]. Interestingly, in contrast to yeast expressed B7-2 and CTLA-4 which like endogenous mammalian costimulatory molecules appeared as a smear of bands in SDS-PAGE, the truncated B7-1 protein migrated as several distinct bands. This most likely illustrates differences in the type of glycosylation of the yeast expressed proteins. Due to such differences the recombinant proteins might be immunogenic in vivo. The addition of specific inhibitors to the growth medium or the use of yeast mutants which have a defect in their glycosylation machinery and glycosylate heterologous proteins to a much lower degree might help to overcome this potential problem [26].

Costimulation via the B7-CD28 pathway plays a crucial role in the activation of T helper cells and cytotoxic T cells. Both, enhancement or prevention of the costimulatory signal might be beneficial under different pathophysiological conditions. Attempts have been made for example to increase the immunogenicity of tumor cells by providing them with B7 molecules on their surface [27]. Systemic treatment of mice with soluble CTLA-4-Ig fusion protein expressed in COS cells resulted in a remarkable suppression of the T cell response, could prevent autoimmune disease, prolonged the survival of xenogeneic tissue grafts, and diminished graft versus host disease [11–13,28]. Soluble CTLA-4 expressed in *P. pastoris* might provide a novel source for large amounts of this potent immunosuppressive molecule.

Here we have shown that biologically active, soluble lymphocyte receptors and their ligands can be produced at high yields in *P. pastoris*. Our data suggest that the yeast expression system could be a suitable alternative to expression of

costimulatory ligands and receptors in mammalian cells. The recombinant proteins obtained might be useful tools to investigate further the complex interactions of costimulatory ligands and receptors, and their role in T cell activation.

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References

[1] A. Weiss, D.R. Littman, Cell 76 (1994) 263–274.
[2] C.E. Rudd, O. Janssen, Y.C. Cai, A.J. da Silva, M. Raab, K.V. Prasad, Immunol Today 15 (1994) 225–234.
[3] P.S. Linsley, W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, J.A. Ledbetter, J Exp Med 174 (1991) 561–569.
[4] L.E. Marengere, P. Waterhouse, G.S. Duncan, H.W. Mittrucker, G.S. Feng, T.W. Mak, Science 272 (1996) 1170–1173.
[5] C.H. June, J.A. Bluestone, L.M. Nadler, C.B. Thompson, Immunol Today 15 (1994) 321–331.
[6] P.S. Linsley, W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle, J.A. Ledbetter, J Exp Med 173 (1991) 721–730.
[7] M. Azuma, D. Ito, H. Yagita, K. Okumura, J.H. Phillips, L.L. Lanier, C. Somoza, Nature 366 (1993) 76–79.
[8] T. Hara, S.M. Fu, J.A. Hansen, J Exp Med 161 (1985) 1513–1524.
[9] P.S. Linsley, J.L. Greene, P. Tan, J. Bradshaw, J.A. Ledbetter, C. Anasetti, N.K. Damle, J Exp Med 176 (1992) 1595–1604.
[10] P.S. Linsley, et al. J Biol Chem 270 (1995) 15417–15424.
[11] D.J. Lenschow, Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S. Linsley, J.A. Bluestone, Science 257 (1992) 789–792.
[12] H. Lin, S.F. Bolling, P.S. Linsley, R.Q. Wei, D. Gordon, C.B. Thompson, L.A. Turka, J Exp Med 178 (1993) 1801–1806.
[13] B.K. Finck, P.S. Linsley, D. Wofsy, Science 265 (1994) 1225–1227.
[14] M. de Boer, L. Conroy, H.Y. Min, J. Kwekkeboom, J Immunol Methods 152 (1992) 15–23.
[15] C. Wulfing, A. Pluckthun, J Mol Biol 242 (1994) 655–669.
[16] K.B. Fryxell, K. O'Donoghue, R.M. Graeff, H.C. Lee, W.D. Branton, Protein Express Purif 6 (1995) 329–336.
[17] A. Kalandadze, M. Galleno, L. Foncerrada, J.L. Strominger, K.W. Wucherpfennig, J Biol Chem 271 (1996) 20156–20162.
[18] K. Busser, E. Garon, D. Higgins, Am Biotech Lab 11 (1993) 10–12.
[19] G.I. Evan, G.K. Lewis, G. Ramsay, J.M. Bishop, Mol Cell Biol 5 (1985) 3610–3616.
[20] S.W. Lee, M.L. Kahn, D.A. Dichek, J Biol Chem 267 (1992) 13020–13027.
[21] C.A. Scorer, J.J. Clare, W.R. McCombie, M.A. Romanos, K. Sreekrishna, Bio/Technology 12 (1994) 181–184.
[22] K.A. Barr, S.A. Hopkins, K. Sreekrishna, Pharmacol Eng 12 (1992) 48–51.
[23] J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, Curr. Prot. Immunol. 2 (1993) 3.2.1–3.2.3.
[24] O. Leo, M. Foo, D.H. Sachs, L.E. Samelson, J.A. Bluestone, Proc Natl Acad Sci USA 84 (1987) 1374–1378.
[25] P.S. Linsley, J.L. Greene, W. Brady, J. Bajorath, J.A. Ledbetter, R. Peach, Immunity 1 (1994) 793–801.
[26] L. Lehle, A. Eiden, K. Lehnert, A. Haselbeck, E. Kopetzki, FEBS Lett 370 (1995) 41–45.
[27] E.B. Brunschwig, E. Levine, U. Trefzer, M.L. Tykocinski, J Immunol 155 (1995) 5498–5505.
[28] P.S. Linsley, P.M. Wallace, J. Johnson, M.G. Gibson, J.L. Greene, J.A. Ledbetter, C. Singh, M.A. Tepper, Science 257 (1992) 792–795.