Non-glycosylated human B7-1(CD80) retains the capacity to bind its counter-receptors

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Abstract Though the cell surface-associated costimulator B7-1(CD80) is known to be highly *N*-glycosylated, the functional significance of this *N*-glycosylation has not been evaluated. Two experimental approaches were taken to assess the influence of *N*-glycosylation on human B7-1 function. First, stable K562 transfectants expressing human B7-1 were treated with the *N*-glycosylation inhibitor tunicamycin. This treatment reduced the levels of B7-1 at the cell surface as judged by both indirect immunofluorescence/flow cytometry and immunoprecipitation analyses. Significantly, the non-glycosylated cell surface-associated B7-1 on tunicamycin-treated cells retained the capacity to bind CTLA-4·Ig, a soluble derivative of the CTLA-4(CD152) counter-receptor. Second, experiments were performed with bacterially-produced non-glycosylated derivatives of human B7-1, comprising either the complete B7-1 extracellular domain (hB7-1·ed) or the membrane-proximal IgC-homologue domain of B7-1 in isolation (hB7-1·IgC). While the hB7-1·IgC derivative failed to bind to CTLA-4, the larger hB7-1·ed derivative associated with CTLA-4·Ig in cell-free binding assays. Futhermore, recombinant hB7-1·ed effectively blocked B7-1-mediated costimulation in an in vitro T cell proliferation assay, suggesting that this soluble non-glycosylated B7-1 derivative is capable of engaging CD28, the B7 counter-receptor implicated in T cell activation. Taken together, these data indicate that the *N*-glycosylation of B7-1 is not required for its association with counter-receptors. Moreover, the findings pave the way for the therapeutic use of recombinant bacterial B7-1 derivatives as competitive inhibitors of B7-mediated signals.

Key words: Human B7-1(CD80); Glycosylation; Bacterial expression; Counter-receptor binding

1. Introduction

T cell activation requires signaling through both antigen and costimulator receptors [1–3]. Although costimulator function has been ascribed to a variety of cell surface proteins [1,4], two of them, B7-1(CD80) and B7-2(CD86), are thought to be especially important [5–8]. Each of these B7 proteins can associate with two structurally-distinct counter-receptors on T cells, CD28 and CTLA-4(CD152), with the former serving as the primary activating receptor [8].

T cell receptor engagement in the absence of costimulation can result in the induction of T cell non-responsiveness [9,10]. This experimental observation has led to attempts to invoke costimulator blockade as a therapeutic means for attenuating pathogenic T cell function [11,12]. A soluble derivative of the CTLA-4 receptor, CTLA-4·Ig, has been studied widely and has shown some therapeutic promise as a competitive inhibitor of B7-driven costimulatory signaling [11–16], although in some animal disease models this agent has proven less effective [17–19]. While this limited efficacy may point to parallel signaling by other costimulators, it may also result from problems inherent to CTLA-4·Ig itself. Alternative agents for blocking the B7/CD28 axis have also been considered. The infusion of anti-B7-1 and anti-B7-2 mAbs in combination was effective in eliminating GVHD lethality in one animal model [20], but studies in other in vivo systems have not shown similar efficacy for anti-B7 mAbs [16,21,22], possibly as a consequence of host responses to the infused antibodies [23]. In principle, recombinant soluble B7 protein derivatives might also be used as competitive inhibitors of the B7/CD28 signaling axis. However, B7·Ig fusion proteins (produced in mammalian expression systems) [24–26] and B7 extracellular domain derivative proteins (produced in yeast expression systems) [27] have not been invoked for the purpose of competitive blockade of T cell activation. To date, there have been no reports on bacterially-produced B7 proteins, and hence there is no indication as to whether non-glycosylated recombinant B7 proteins of this sort might retain counter-receptor affinity, and hence competitive inhibitory potential.

Human B7-1 and B7-2 are type I membrane proteins, each with an extracellular domain consisting of one IgV-homologue domain unit linked in tandem to one IgC-homologue domain unit. A prominent structural feature of the B7 proteins is their *N*-glycosylation [6], with human B7-1 sequence displaying eight potential acceptor sites for *N*-linked glycosylation [5]. However, the extent of usage of each site has not been established, and the role of glycosylation in B7 activities has not been systematically investigated.

In the present study, the contribution of *N*-glycosylation to the ability of human B7-1 to associate with its counter-receptors is evaluated. The complementary experimental approaches in this study invoke non-glycosylated B7-1 on tunicamycin-treated mammalian cell transfectants on the one hand, and recombinant soluble B7-1 proteins produced in bacterial cell transformants on the other. The possibility of using bacterial B7 protein derivatives, reported here for the first time, as therapeutic agents emerges.

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Abbreviations: hB7-1, human B7-1; hB7-1·IgC, human B7-1 immunoglobulin constant region homology domain; hB7-1·IgV, human B7-1 immunoglobulin variable region homology domain; hB7-1·ed, human B7-1 extracellular domain

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2. Materials and methods

2.1. Mammalian cell transfectants

Full-length coding sequence for human B7-1 was mobilized from the plasmid phB7-1/cEXV-3 (provided by J. Miller, University of Chicago) [28] by digesting with *Eco*RI, and the insert was ligated into the corresponding site of pBT (Bluescript; Stratagene, Inc., La Jolla, CA), generating phB7-1/BT. The full-length cDNA was then excised from phB7-1/BT with *Bam*HI and *Hin*dIII and was subcloned into the corresponding sites of our Epstein-Barr virus episomal expression vector pREP10 (distributed by Invitrogen Co., San Diego, CA), generating the expression construct phB7-1/REP10.

phB7-1/REP10 was introduced into K562 myeloid leukemic cells (American Type Culture Collection, Rockville, MD) by lipofection as described [29]. Briefly, K562 cells were lipofected in serum-free medium using 30 μl of lipofectin reagent (Life Technologies, Grand Island, NY) and 10 μg of plasmid DNA. Cells were selected in the presence of 200 μg/ml of hygromycin B (Calbiochem-Novabiochem Co., La Jolla, CA). Drug-resistant cells were pooled and used uncloned.

2.2. Production of recombinant bacterial proteins

Oligonucleotides used for generating B7 expression constructs by PCR were as follows: primer A: 5′-AGCGAATTCGGTCTTTCTC-ACTTCTGTTCAGGT-3′; primer B: 5′-TATGCGGGATCCTTAG-TGGTGGTGGTGGTGGTGAAGCTTGT TATCAGGAAAATGC-TCTTG-3′; primer C: 5′-TAGCGAATTCAAAGCTGACTTCCCT-ACACCT-3′; primer D: 5′-ACTGGCTAAAGCTTTTATTGCTTG-GTTGTATTCCAGTTG-3′; primer E: 5′-TAGCGAATTCGGTCT-TTCTCACTTCTGTTCAGGT-3′; primer F: 5′-ATTAGAATAAG-CTTTTAGACTGATAACGTCACTTCAGCCAG-3′.

A bacterial expression construct for human B7-1 was produced in a step-wise fashion, with a Pichia expression construct as an intermediate. To start, a cDNA sequence encompassing the complete extracellular domain of human B7-1 (Gly^{1}–Asn^{216}) was generated by PCR using phB7-1/BT as template, and primers A and B as 5′- and 3′-end primers, respectively. The PCR product was co-digested with *Eco*RI and *Bam*HI and ligated into the corresponding sites of the Pichia expression vector pHIL-S1 (Invitrogen Co., San Diego, CA), generating phB7-1/HIL-S1. The construct was verified by DNA sequencing at this stage. In turn, the hB7-1 coding sequence was mobilized from phB7-1/HIL-S1 by *Eco*RI and *Hin*dIII co-digestion, and the recovered insert was ligated into the corresponding sites of the bacterial expression vector pHH/ET22b, generating phB7-1·ed·HH/ET22b. The vector pHH/ET22b had previously been derived by us [30] from pET-22b(+) (Novagen).

A cDNA sequence encompassing only the membrane-proximal IgC domain (Lys^{113}–Gln^{210}) of human B7-1 was generated by PCR using phB7-1/BT as template, and primers C and D as 5′- and 3′-end primers, respectively. The PCR product was co-digested with *Eco*RI and *Hin*dIII and ligated into the corresponding sites of pHH/ET22b, generating phB7-1·IgC·HH/ET22b. In similar fashion, a cDNA sequence encompassing only the IgV domain (Gly^{1}–Val^{112}) of human B7-1 was generated by PCR, in this case using phB7-1/BT as template, and primers E and F as 5′- and 3′-end primers, respectively. The PCR product was co-digested with *Eco*RI and *Hin*dIII and ligated into the corresponding sites of pHH/ET22b, generating hB7-1·IgV·HH/ET22b. The coding sequences within both PCR-derived expression constructs were verified by DNA sequencing.

The bacterial expression constructs were separately transformed into *E. coli* BL21(DE3). Ampicillin-resistant single colonies were picked and assessed for B7 protein expression after induction by 1 mM IPTG. Quantitative amounts of the proteins were prepared in one-liter batches. hB7-1·IgC was purified from the periplasm of bacterial transformants according to the manufacturer's protocol (Novagen). Briefly, bacteria were pelleted, washed with PBS, resuspended in 0.4 culture volumes of 30 mM Tris-HCl pH 8.0/20% sucrose. EDTA was added to 1 mM and the suspension was incubated for 5 min at room temperature (RT) with shaking. Bacteria were collected and resuspended in 0.4 culture volumes of ice-cold 5 mM $MgSO_4$. The suspension was incubated for 10 min on ice with shaking. After centrifugation, the supernatant represents the periplasmic fraction. hB7-1·IgC was purified from the periplasmic fraction by Ni^{2+}-Sepharose chromatography.

hB7-1·ed and hB7-1·IgV were purified according to a published protocol [31] with modifications. Briefly, bacterial cells were collected by centrifugation and lysed by treating with lysozyme (1 mg/ml) and sonicating at 4°C. The resulting lysate was centrifuged at $1100\times g$ for 8 min to remove unbroken cells. Inclusion bodies were subsequently collected from the lysate by centrifuging at $25\,000\times g$ for 30 min. Partially purified inclusion bodies (PPIB) were dissolved in 2% sodium lauroylsarcosine (Sigma)/50 mM Tris-HCl, pH 10.0 (500 ml detergent solution per 1 l of starting bacterial culture). Renaturation was performed in the presence of 50 μM copper sulfate at RT for 20 h. After this step, 2 ml of charged His·Bind Sepharose (Novagen) was added (per 500 ml), and batch absorption was conducted over a 16-h period. Protein-bound beads were collected and packed into a column. The column was washed and proteins were eluted with an imidazole buffer according to the manufacturer's protocol (Novagen). The fractions were analyzed by SDS-PAGE, and the protein-containing fractions were combined and dialyzed vs. PBS. Non-covalent aggregates that formed during the dialysis procedure were removed by centrifugation.

2.3. Indirect immunofluorescence/flow cytometry

K562 cells and transfectants were washed twice in wash buffer (0.25% BSA/0.1% (w/v) sodium azide/PBS). 10^6 washed cells were incubated on ice for 1 h with 100 μl of the wash buffer containing 1 μg of hB7-1-specific mAb BB-1 (Becton-Dickinson) or with 100 μl of spent culture medium containing CTLA-4·Ig prepared as described [32]. The cells were washed twice and incubated on ice for 1 h with 50 μl 1:100 diluted FITC-conjugated goat $F(ab')_2$ anti-mouse Ig (for BB-1), or anti-human Ig (for CTLA-4·Ig) (Boehringer Mannheim Biochemicals). The cells were washed twice, fixed in 500 μl of 2% paraformaldehyde/wash buffer, and analyzed on a FACStar (Becton-Dickinson).

2.4. Surface biotinylation and immunoprecipitation

K562 cells were washed three times in ice-cold PBS, pH 8.0 and resuspended at 10^7 cells/ml in 1 mg/ml of sulfo-NHS-biotin (Pierce, Rockford, IL) dissolved in PBS, pH 8.0. The cells were incubated at 4°C for 30 min with gentle mixing. The labeling reactions were quenched by adding serum-free RPMI-1640. The excess biotin was removed by washing cells four times with PBS. The surface-biotinylated cells (5×10^7) were lysed in 1 ml of 1% Nonidet P40/150 mM NaCl/50 mM Tris-HCl, pH 8.0/1 mM PMSF at 4°C for 1 h. The lysate was spun in a microfuge for 30 min to remove debris, precleared with protein G-agarose (Gibco-BRL), and immunoprecipitated with 60 μl of protein G-agarose slurry (50%) and 1 μg of BB-1 or 2 μg of CTLA-4·Ig at 4°C for 16 h. Beads were washed, spun over a sucrose gradient, and washed again as described [30]. The immunoprecipitates were fractionated on a 10% SDS-polyacrylamide gel and electro-transferred to nitrocellulose membrane. The membrane was blocked in 3% BSA/PBS, washed with 0.5% BSA/T-PBS (0.1% Tween-20 in PBS), and incubated with streptavidin-horseradish peroxidase conjugate (Amersham Co., Arlington Heights, IL) diluted in 1% BSA/T-PBS. The membrane was washed in T-PBS, developed with enhanced chemiluminescence reagent (ICN Biomedicals, Inc., Irvine, CA), and exposed to X-ray film.

2.5. Solid-phase binding assays

In one type of binding assay, test or control protein was bound to Immulon-4 wells (Dynatech, Chantilly, VA) at 10 μg/ml in 100 μl of PBS for 4 h at 37°C. Wells were washed with wash buffer (0.5% BSA/0.05% Tween-20/PBS) and blocked with 200 μl blocking buffer (3% BSA/0.05% Tween-20/PBS) for 16 h at 4°C. Wells were washed and incubated for 2 h at 37°C with 100 μl of 10 μg/ml CTLA-4·Ig diluted in the wash buffer. Plates were washed and then incubated for 2 h at 37°C with 100 μl of 1:800 diluted HRP-conjugated anti-human IgG1 Ab (The Binding Site Ltd, Birmingham, UK). Plates were washed, developed, and analyzed as described [30]. All experiments were performed in triplicate.

Alternatively, 1 μg of CTLA-4·Ig was bound to Immulon-4 for 4 h at 37°C. Wells were washed, blocked, and incubated for 4 h at 37°C with 100 μl of 10 μg/ml bacterial protein diluted in PBS. Plates were washed and incubated for 2 h at 37°C with the anti-hemagglutinin (HA) antibody 12CA5 (provided by D. Templeton, CWRU) at a final concentration of 0.25 μg/ml in the wash buffer. Wells were washed and incubated for 2 h at 37°C with 100 μl 1:2000 diluted HRP-conjugated goat anti-mouse Ig (BioRad). Plates were washed, developed, and analyzed. All experiments were performed in triplicate.

2.6. CTLA-4·Ig-agarose bead binding assay

CTLA-4·Ig was cross-linked to protein G-agarose beads as described [33]. hB7-1·ed at 100 μg/ml in PBS was mixed with CTLA-4·Ig-conjugated beads for 16 h with gentle mixing at 4°C. The beads were collected, packed into a column, and washed with PBS. hB7-1·ed was eluted from the beads with 100 mM glycine, pH 2.5 in 0.5-ml fractions and immediately neutralized with 200 mM Na_2HPO_4.

2.7. Costimulation assays

Peripheral blood mononuclear cells (PBMC) were isolated from fresh whole blood by Ficoll density centrifugation and depleted of adherent cells by incubation in plastic flasks for 1 h at 37°C. T cells were purified by two rounds of treatment with Lympho-kwik (One Lambda, Inc., Canoga Park, CA). T cell purity was verified by the lack of a T cell proliferative response to PHA or PMA in the absence of accessory cells. To analyze proliferation, 1×10^5 T cells were incubated in flat-bottom 96-well plates with 1 ng/ml PMA and mitomycin C treated hB7-1-expressing K562 transfectant cells (8×10^4 in Fig. 6, panel A and 2×10^4 in Fig. 6, panel B) for 60 h. Wells were pulsed with 1 μCi ^{3}H-thymidine for the last 16 h of the incubation period. Cells were harvested and counted on a Betaplate liquid scintillation counter.

In some inhibition experiments, CTLA-4·Ig or control mCD8·Ig was included in the assays at a final concentration of 10 μg/ml. In other inhibition experiments, the immunoaffinity-purified bacterial hB7-1·ed (or control bacterial protein) was included in the assays at a final concentration of 1, 3, 9, or 27 μg/ml.

3. Results

3.1. Non-glycosylated B7-1 on the surface of tunicamycin-treated K562 transfectant cells binds to CTLA-4·Ig

In a first set of studies, the B7-1 *N*-glycosylation issue was addressed in human cell transfectants. To this end, the human myeloid leukemia cell line K562 was stably transfected with the episomal expression construct phB7-1/REP10. Surface expression of human B7-1 on the transfectants was documented by immunoprecipitation of cell surface-biotinylated cell lysates. While parental K562 cells lacked detectable B7-1 (Fig. 1, lanes 1 and 3), transfected K562 cells expressed cell surface B7-1 that could be readily immunoprecipitated with either the anti-B7-1 mAb BB-1 (Fig. 1, lane 2) or CTLA-4·Ig (Fig. 1, lane 4). The observed molecular weight of ~66 kDa agrees with the predicted size of glycosylated human B7-1 [5]. The

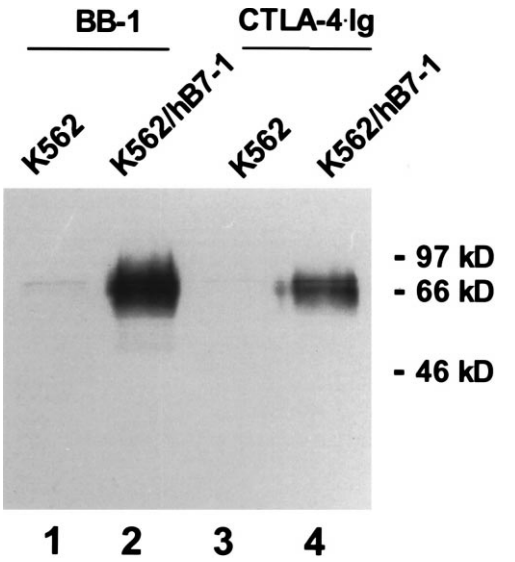

Fig. 1. Surface expression of hB7-1 on K562 transfectant cells. Non-transfected (lanes 1 and 3) or phB7-1/REP10-transfected (lanes 2 and 4) K562 cells were cell surface-biotinylated, and cell lysates were immunoprecipitated using the human B7-1-specific mAb BB-1 (lanes 1 and 2) or CTLA-4·Ig (lanes 3 and 4). Immunoprecipitates were fractionated by SDS-PAGE and developed as described in Section 2.

effect of tunicamycin, an inhibitor of *N*-linked glycosylation, on B7-1 expression in the K562 transfectants was next evaluated. Transfectants treated with tunicamycin at 10 μg/ml for 40 h displayed significantly decreased levels of B7-1 at their cell surfaces as compared to the same transfectants treated instead with the solvent DMSO only (Fig. 2, panels A and B). The reduction in surface B7-1 (as evaluated by immunofluorescence and flow cytometry) was evident regardless of whether an anti-B7-1 mAb (Fig. 2, panel A) or CTLA-4·Ig (Fig. 2, panel B) was used as the detecting reagent.

To more fully characterize the B7-1 protein on the surfaces of tunicamycin-treated K562 transfectants, the protein was immunoprecipitated from surface-biotinylated cell lysates.

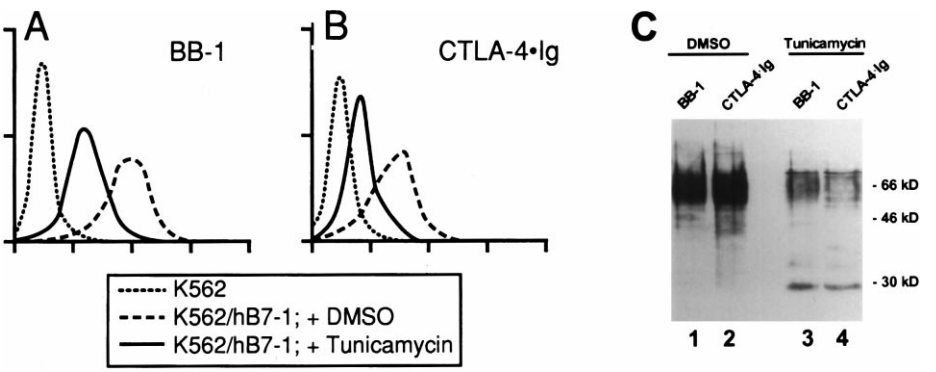

Fig. 2. CTLA-4·Ig binds to non-glycosylated B7-1 of tunicamycin-treated mammalian cell transfectants. Panels A and B: phB7-1/REP10-transfected K562 cells were cultured in the presence of tunicamycin (10 μg/ml in DMSO solvent) (solid line) or the DMSO solvent only (dashed line) for 40 h. The treated cells (solid and dashed lines) or control cells (dotted line) were immunostained with BB-1 mAb (panel A) or CTLA-4·Ig (panel B). FITC-conjugated goat anti-mouse Ig (BB-1) or goat anti-human Ig (CTLA-4·Ig) were used as secondary Abs. Cells were analyzed on a FACScan flow cytometer and plotted as arbitrary units of $\log_{10}$ fluorescence intensity vs. cell number. Panel C: The same phB7-1/REP10-transfected K562 cells were cultured in the presence of tunicamycin (DMSO solvent) (lanes 3 and 4) or the DMSO solvent only (lanes 1 and 2) for 40 h. In each case, 5×10^7 cells were surface-biotinylated, and cell lysates were immunoprecipitated using BB-1 mAb (lanes 1 and 3) or CTLA-4·Ig (lanes 2 and 4). Immunoprecipitates were fractionated by 10% SDS-PAGE and blotted onto nitrocellulose. Biotinylated proteins were detected by streptavidin-HRP followed by ECL and fluorography.

Two distinct populations of B7-1 molecules were evident. A subset of the B7-1 protein from the tunicamycin-treated cells co-migrated (at ~66 kDa) with the B7-1 protein of control DMSO-treated cells (Fig. 2, panel C), indicating that it had retained its *N*-glycosylation despite tunicamycin treatment. However, a second 30-kDa species was also clearly evident in the tunicamycin-treated, but not the control DMSO-treated, samples. The size of this protein species corresponds to that predicted (based upon amino acid composition) for non-glycosylated human B7-1 protein. Significantly, this non-glycosylated B7-1 species was immunoprecipitated not just by mAb BB-1 (Fig. 2, panel C, lane 3) but also by the soluble B7 counter-receptor CTLA-4·Ig (Fig. 2, panel C, lane 4). This finding suggests that *N*-glycosylation is not necessary for human B7-1 to bind to its counter-receptor CTLA-4.

It is noteworthy that consistent with the previous flow cytometric finding, the immunoprecipitated samples showed significantly lower B7-1 levels on tunicamycin-treated cell surfaces (Fig. 2, panels A and B). When immunoprecipitation was performed using biotinylated total cell lysates instead of surface biotinylated ones, B7-1 levels were not affected by tunicamycin treatment (data not shown). These findings indicate that tunicamycin does not inhibit B7-1 protein synthesis per se and suggest that tunicamycin treatment may inhibit the export and/or diminish the cell surface stability of B7-1 on transfected cell surfaces. Of note, the B7-1 decrease is evident for both the glycosylated and non-glycosylated species. In addition, the recovery of non-glycosylated B7-1 from surface biotinylated samples suggests that glycosylation is not an absolute prerequisite for B7-1 exit to the cell surface.

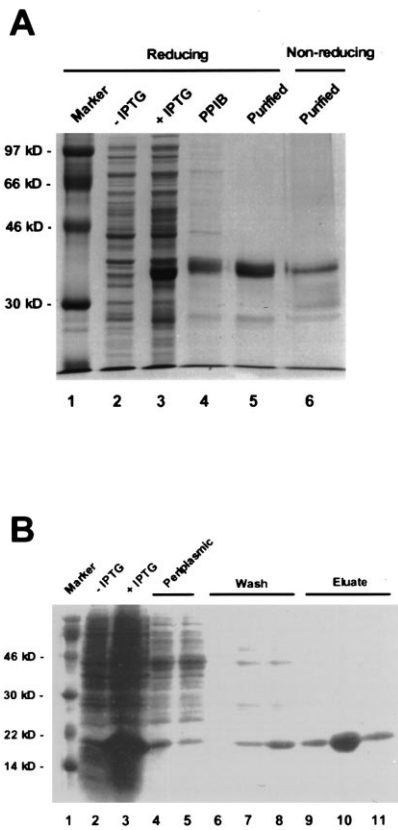

Fig. 3. Purification of bacterially-expressed soluble human B7-1 protein derivatives. Panel A: Recombinant human B7-1·ed (full extracellular domain) protein was purified from extracts of bacteria transformed with phB7-1·ed·HH/ET22b. Protein enrichment during the refolding/purification process was monitored at each stage by 10% SDS-PAGE. Lane 1, molecular mass markers; lanes 2 and 3, total lysates of uninduced and induced cells, respectively; lane 4, partially purified inclusion bodies; lanes 5 and 6, pooled 1 M imidazole eluates from the Ni^{2+}-metal affinity column. Samples were run under reducing conditions except for lane 6. Panel B: Recombinant human B7-1·IgC (IgC-homologue domain only) protein was purified from extracts of bacteria transformed with phB7-1·IgC·HH/ET22b. Protein enrichment during the refolding/purification process was monitored at each stage by 15% SDS-PAGE. Lane 1, molecular mass markers; lanes 2 and 3, total lysates of uninduced and induced cells, respectively; lanes 4 and 5, periplasmic preparations before and after incubation with His·Bind-Sepharose, respectively; lanes 6–8, flow-through during washing stage; lanes 9–11, fractions 1–3 during imidazole elution of bound protein. Samples were run under reducing conditions.

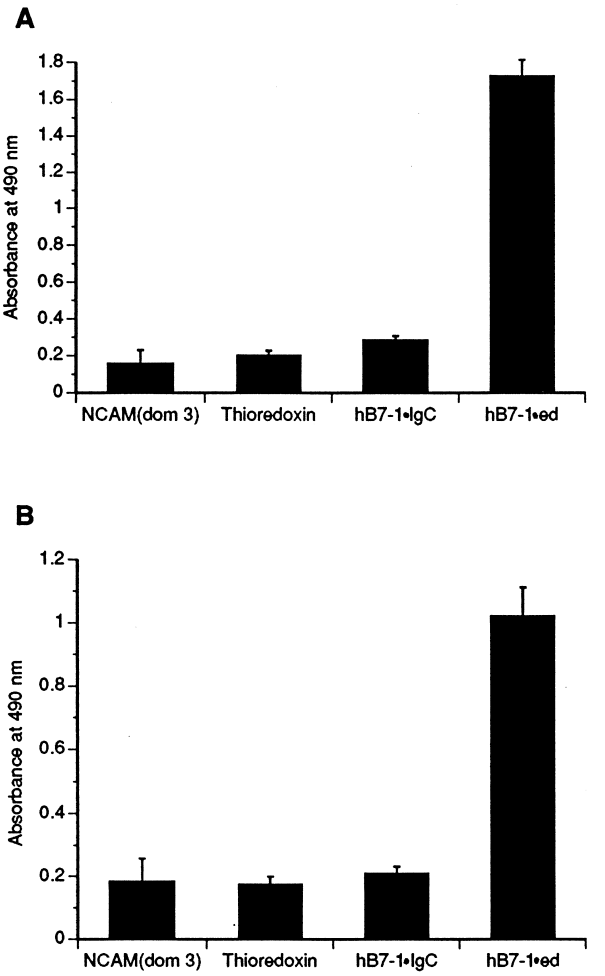

Fig. 4. Plate-based solid phase binding assay demonstrating specific interaction between recombinant hB7-1·ed and CTLA-4·Ig proteins. Panel A: hB7-1·ed protein (10 μg/ml), purified by metal chelate affinity chromatography, was pre-coated onto wells of Immulon-4 plates, and CTLA-4·Ig (10 μg/ml) was added to the wells. After the washing step, immobilized CTLA-4·Ig was detected using HRP-conjugated anti-human IgG1 Ab. Panel B: The binding partners were reversed (as compared to panel A) so that CTLA-4·Ig (20 μg/ml) was pre-coated onto the wells, and hB7-1·ed (10 μg/ml) binding was monitored. Bound hB7-1·ed was detected using the anti-hemagglutinin mAb 12CA5 as primary Ab and HRP-conjugated goat anti-mouse Ig antiserum as secondary Ab. Bars indicate the mean absorbance and standard error of triplicate samples. The results are representative of at least two independent experiments with similar results.

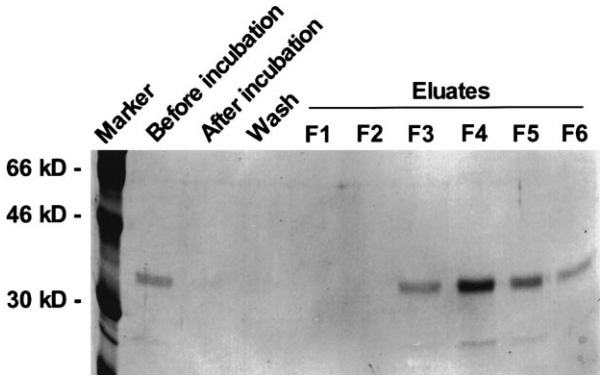

Fig. 5. Binding of hB7-1·ed to CTLA-4·Ig-conjugated protein G-agarose beads. hB7-1·ed protein (200 μg/ml) was co-incubated for 16 h with CTLA-4·Ig-conjugated protein G-agarose beads. The beads were packed into a column and extensively washed with PBS, and bound hB7-1·ed was eluted in 0.5-ml fractions with 100 mM glycine, pH 2.5 and neutralized with 200 mM Na_2HPO_4. Fractions were analyzed on a 10% SDS-PAGE. Lane 1, molecular mass markers; lane 2, protein sample before the addition of the beads; lane 3, the same amount of protein as in lane 2 after incubation with the beads; lane 4, flow-through during the washing stage; lanes 5–10, fractions 1–6 during 100 mM glycine (pH 2.5) elution.

3.2. Production of human B7-1 derivatives in a bacterial expression system

To address the *N*-glycosylation question in a more direct way, we shifted from the mammalian expression system to a bacterial expression system which produces non-glycosylated recombinant proteins. Bacterial expression of B7 proteins has not been reported previously. To this end, we generated the expression construct phB7-1·ed·HH/ET22b which contains the coding sequence for the full extracellular domain of human B7-1 (designated hB7-1·ed), along with hexahistidine and hemagglutinin epitope tags. Specific accumulation of an ~32-kDa band was observed after IPTG induction (Fig. 3, panel A, lanes 2 and 3). While the induced cells expressed high levels of recombinant protein, periplasmic soluble protein yields were poor because the protein formed inclusion body aggregates (Fig. 3, panel A, lane 4). The inclusion bodies were dissolved, refolded, and purified as described in Section 2. After purification by nickel-chelate affinity chromatography, hB7-1·ed appeared as a dominant band under both reducing (Fig. 3, panel A, lane 5) and non-reducing (Fig. 3, panel A, lane 6) conditions, indicating that the protein is a monomer under SDS-PAGE conditions. The refolded hB7-1·ed protein, but not control protein thioredoxin, bound to the B7-1-specific mAb BB-1 in a concentration-dependent manner (data not shown). Overall, the purification/refolding efficiency for hB7-1·ed was 30%, with approximately 2 mg purified protein obtained per liter of bacterial culture.

In addition to hB7-1·ed, two truncated derivatives of the human B7-1 extracellular domain were produced in the same bacterial expression system for inclusion in the subsequent counter-receptor binding analyses. These derivatives consisted of the membrane-proximal IgC and amino-terminal IgV domains of hB7-1, each on its own as isolated domain units, and were produced in *E. coli* transformed with the expression constructs phB7-1·IgC·HH/ET22b and phB7-1·IgV·HH/ET22b, respectively (see Section 2). In the case of phB7-1·IgC·HH/ET22b transformants, significant accumulation of an ~20-kDa band was observed after IPTG induction (Fig. 3, panel B, lane 3). Moreover, there was substantial secretion of this hB7-1·IgC protein in soluble form into the bacterial periplasm, contrasting with hB7-1·ed which accumulated in insoluble inclusion bodies and did not efficiently exit into the periplasm. The periplasmic hB7-1·IgC protein could be highly purified by single-step metal chelate chromatograpy (Fig. 3, panel B, lanes 9–11). Though no Ab with specificity for this isolated domain is available, the efficient secretion of hB7-1·IgC into the bacterial periplasm suggests that this protein folds properly within the bacterial environment, and as shown, migrates as expected at ~20 kDa. Generally, approximately 5 mg of periplasmic hB7-1·IgC could be purified from one liter of bacterial culture. In contrast, repeated attempts to express the human hB7-1·IgV protein were un-

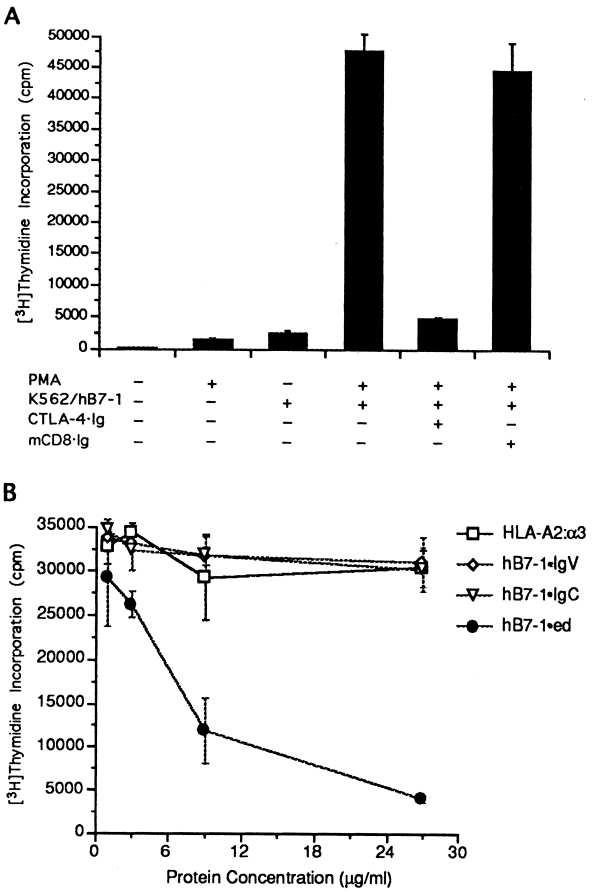

Fig. 6. Soluble bacterially-produced human hB7-1·ed competitively inhibits B7-1-dependent costimulation. Panel A: 10^5 purified human T cells were combined with PMA (1 ng/ml) and 8×10^4 mitomycin C-treated phB7-1/REP10-transfected K562 cells. Effective depletion of accessory cells was documented in all T cell preparations by demonstrating the lack of a response to PMA or PHA in the absence of a source for costimulation. For inhibition of the costimulation, CTLA-4·Ig or control Ig was added to the cultures at a final concentration of 10 μg/ml. All cultures were incubated for 60 h and pulsed with 1 μCi of [^{3}H]thymidine per well for the last 16 h of the incubation period prior to determining [^{3}H]thymidine incorporation. Points are the means and standard errors of triplicate samples. The data are representative of at least three independent experiments with similar results. Panel B: 10^5 purified human T cells were combined with PMA (1 ng/ml) and 2×10^4 mitomycin C-treated phB7-1/REP10-transfected K562 cells. hB7-1·ed or control proteins at final concentrations of 1, 3, 9, or 27 μg/ml were included in the costimulation assays (as characterized in panel A). Points are the means and standard errors of triplicate samples. The data are representative of at least two independent experiments with similar results.

successful, with the protein aggregating in inclusion bodies and failing to reconstitute the BB-1 mAb-interactive epitope hat resides within this domain after the refolding procedure.

3.3. Non-glycosylated bacterially-produced human B7-1 binds to CTLA-4

Two types of solid-phase binding assays were employed for assessing the binding of soluble non-glycosylated B7-1 derivatives to CTLA-4. First, CTLA-4·Ig binding to immobilized B7-1 vs. control proteins was evaluated, using HRP-conjugated anti-human IgG1 as a detecting reagent. Control proteins for these experiments included recombinant isolated IgSF domain proteins (domain 3 of mouse NCAM and the α_3 domain of HLA-A2.1) produced with the same bacterial expression vectors, as well as BSA and bacterial thioredoxin (TX) produced with the pET-32a(+) vector. As shown in Fig. 4 (panel A), CTLA-4·Ig bound to hB7-1·ed, but not to hB7-1·IgC or any of the negative control proteins (data from a separate experiment with the HLA-A2.1 α_3 domain and BSA negative control proteins not shown).

Second, a sandwich ELISA format was used in which immobilized CTLA-4·Ig served to capture B7 derivatives which were then detected with an mAb directed against the HA epitope tag and HRP-conjugated goat anti-mouse Ig. Consistent with the findings in the other ELISA, non-glycosylated hB7-1·ed bound to CTLA-4·Ig, whereas no significant binding was observed for the truncated hB7-1·IgC or the control proteins (Fig. 4, panel B).

An alternative assay was also employed that bypassed Immulon-4 wells altogether. In this case, CTLA-4·Ig was first cross-linked to protein G-agarose, and the CTLA-4-conjugated beads were then shown to efficiently adsorb the hB7-1·ed protein (Fig. 5, lanes 2 and 3). Extensive washing did not visibly release bound protein from the affinity matrix (Fig. 5, lane 4). The hB7-1·ed protein was readily eluted from the beads with 100 mM glycine, pH 2.5 (Fig. 5, lanes 7–10). Taken together, these results establish that glycosylation is not required for B7-1/CTLA-4 interaction.

3.4. Non-glycosylated bacterial hB7-1·ed is functional as a competitive inhibitor of cell surface B7-1-mediated costimulation

We next determined whether the non-glycosylated B7-1 proteins expressed in *E. coli* can bind to the primary activating counter-receptor on T-cells, CD28. To this end, we devised an assay paralleling one previously reported in which the capacity of mutant B7·Ig derivatives to block immobilized B7·Ig-mediated costimulation of T cell proliferation was assessed as an indicator of CD28 engagement [26]. In our case, human T cells were stimulated to proliferate with a suboptimal dose of the phorbol ester PMA and hB7-1-expressing K562 transfectants in combination, providing the primary and costimulatory signals, respectively. As expected, no proliferation was observed in the presence of untransfected or vector only-transfected K562 cells (data not shown), nor in the absence of stimulator cells altogether (Fig. 6, panel A). However, addition of hB7-1 K562 transfectant cells yielded significant T-cell proliferation (Fig. 6, panel A), and the B7-dependence of this costimulatory effect was substantiated by its complete inhibitability by CTLA-4·Ig and not a control Ig fusion protein. With the costimulation assay in place, we asked whether soluble non-glycosylated (monomeric) B7-1 derivatives could competitively block costimulation by B7-1 on the surface of K562 transfectants. Significantly, hB7-1·ed blocked T cell proliferation in a dose-dependent manner, with near total inhibition achieved at $\sim$25 μg/ml of hB7-1·ed (Fig. 6, panel B). Similar levels of costimulatory blockade were achieved with 10 μg/ml CTLA-4·Ig. In contrast, hB7-1·IgC and control proteins displayed no inhibitory effects. Taken together, the results demonstrate that the non-glycosylated hB7-1 (but not the IgC domain derivative) retains the ability to bind to CD28 and thereby inhibit T cell proliferation in vitro.

4. Discussion

Though B7 proteins are known to be highly *N*-glycosylated, the relevance of this glycosylation to function has remained an open question. In the present study, we have used mammalian and bacterial expression systems in combination to address this issue. Our principal findings are: (1) Non-glycosylated cell surface-associated B7-1, as expressed on tunicamycin-treated human cell transfectants, retains the capacity to engage the counter-receptor CTLA-4. (2) A soluble derivative of human B7-1 consisting of the complete extracellular domain can be produced in bacterial cell transformants and refolded in functional form. (3) This non-glycosylated, refolded B7-1 produced in bacteria can bind to a soluble derivative of CTLA-4 in cell-free binding assays and can inhibit costimulation dependent upon B7/CD28 interaction in a cellular proliferation assay. (4) A truncated derivative of B7-1 consisting of the IgC homologue domain in isolation (free of the amino-terminal IgV homologue domain, as well as the transmembrane and cytoplasmic domains), though readily produced and efficiently secreted into the bacterial periplasm, cannot interact with the B7 counter-receptors. Taken together, the data establish that *N*-glycosylation is not obligatorily required for human B7-1 binding to its counter-receptors, and furthermore raise the possibility of using bacterially-produced B7-1 derivatives consisting of the complete B7-1 extracellular domain as therapeutic blockers.

Our findings fit well with two other reports that have similarly argued, albeit in a more indirect way, against the likelihood of an obligatory role for *N*-glycosylation in B7-1 function. Based on three-dimensional computer-generated models of the human B7-1 IgV- and IgC-like domains, Peach et al. hypothesized that all of the potential *N*-linked glycosylation sites in the IgV and IgC domains are positioned in regions that are essentially opposite the predicted site of counter-receptor interaction, and are therefore unlikely to modulate the interaction [25]. Fargeas et al. expressed on L cells a panel of site-specifically mutated human B7-1 proteins, a subset of which had one or two *N*-glycosylation sites removed. One mutant, M22, yielded a two-fold increase in binding to $CD28^+$ Jurkat cells, and the remaining glycosylation-site mutants exhibited no significant perturbation in binding [34]. Our data, based upon use of a glycosylation inhibitor and non-glycosylated recombinant bacterial derivatives, complements these observations. Fargeas et al. mutated only a subset of the *N*-glycosylation sites within B7-1 and, even for these, just one or two sites were mutated at a time. Our findings demonstrate that *N*-glycosylation can be fully eliminated without loss of CTLA-4 and CD28 binding, solidifying the con-

clusion that B7-1 can fold and bind receptor without *N*-glycosylation.

Though *N*-glycosylation is apparently not obligatorily required for counter-receptor interaction, it may nonetheless play a role in modulating B7 activity and/or in enhancing the surface expression and solubility of native B7-1 and its recombinant derivatives. With respect to the latter, it is noteworthy that whereas B7-1·Ig fusion proteins produced in eukaryotic cells are very soluble [24–26], a recombinant B7-1 protein encompassing the same complete B7-1 extracellular domain sequence is insoluble in our hands when expressed in *E. coli*. This insolubility may be due to the lack of carbohydrates to stabilize protein conformation. Of note, B7 proteins bear a number of hydrophobic residues on the surfaces of their extracellular domains which could affect solubility. In addition, *N*-glycosylation of native transmembrane B7-1 may facilitate its export to the cell surface. Our results in fact indicate that tunicamycin-treated human B7-1-transfected K562 cells express significantly lower levels of B7-1 on their surfaces. The experiments in the present report, however, were not designed to address these issues, and further analysis will be required to complete the picture.

The B7-1 extracellular domain consists of one IgV- and one IgC-homologue domain arrayed in tandem. Despite several studies, the respective contributions of these Ig superfamily domains to counter-receptor binding remain unresolved, with the literature consisting of a mixture of human and murine B7 analyses and some conflicting findings. Inobe et al. showed that an alternatively-spliced form of mouse B7-1 composed of the IgV domain alone binds to both CD28 and CTLA-4 [35]. This finding is consistent with Fargeas et al.'s site-specific mutational analysis which identified critical sites for counter-receptor binding within the human B7-1 IgV-homologue domain [34]. In contrast, Guo et al. reported, in direct conflict with Inobe et al., that an alternatively-spliced form of mouse B7-1 consisting of only the IgV-homologue domain failed to bind to the counter-receptors. These authors concluded that the IgC-homologue domain, but not the IgV-homologue domain, of B7-1 constitutes the binding site [36]. By using soluble human B7·Ig fusion proteins, Peach et al. demonstrated that residues of both IgV and IgC domains are critical for binding [25]. More recently, Ellis et al. demonstrated that the IgV domain of human B7-2 (CD86), but not that of B7-1 (CD80), is sufficient for CTLA-4 binding [26]. In the present report, we have taken a different experimental approach to the question, showing for the first time that an isolated hB7-1·IgC domain, which can be produced abundantly in soluble form in bacterial periplasm, is incapable of engaging either of the B7 counter-receptors. Hence, the data agree with those who argue for either an exclusive role for the IgV domain or for cooperative interactions between the IgV and IgC domains. Of note, the IgV domain, unlike the IgC domain, was not efficiently secreted into bacterial periplasm, and when refolded under our conditions, did not bind to counter-receptors (not shown). In this case, however, one cannot rule out refolding aberrancies.

Gerstmayer et al. recently reported the successful production of soluble human B7-1 and B7-2 derivatives in a yeast expression system [27]. These investigators were apparently unable to refold functional B7 protein derivatives from bacteria, but experimental details were not provided. In contrast, we have succeeded in efficiently refolding a soluble human B7-1 derivative, with high yields of the refolded protein (approximately 2 mg/l culture). This represents the first report of a soluble recombinant B7 protein derivative produced in bacteria. A key to our B7-1 refolding success may be our application of a refined refolding procedure which incorporates the detergent sodium lauroylsarcosine into the denaturation/renaturation buffer and omits guanidine or urea denaturants [31]. This procedure, which was applied to single chain Fv refolding in the previous study, may be especially well-suited for the refolding of IgSF protein derivatives in general and appears to be effective in settings where guanidine/urea denaturants fail.

Functional recombinant B7-1 derivatives have a variety of potential experimental and therapeutic applications. On the experimental side, non-glycosylated bacterial B7-1 derivatives, when used in combination with cell-free binding assays, provide a simplified means for probing structure/function correlations and determinants of counter-receptor affinity. This type of cell-free analysis avoids confounding factors associated with the use of whole cells bearing B7 proteins in traditional binding assays. We have previously shown that another cell surface molecular interaction of immune relevance, namely that between CD8 and MHC class I, can be effectively modeled by using soluble domain derivatives of the respective proteins [30,37]. On the therapeutic side, recombinant B7 protein derivatives might be used as competitive blockers of B7-driven, CD28-mediated costimulation. These would provide alternatives to CTLA-4·Ig which could, by contrast with CTLA-4·Ig, be produced by more economical bacterial (vs. mammalian) expression systems. Numerous protein engineering options for recombinant B7-1 derivatives can now be considered and experimentally evaluated.

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