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Primary Structure and Functional Characterization of a Soluble, Alternatively Spliced Form of B7-1


Recent studies have suggested that soluble forms of B7-1 and B7-2 may exist, but transcripts that code for these molecules have not been previously described. In this study, we report the cloning and characterization of an alternatively spliced soluble form of porcine B7-1 (sB7-1) that lacks exons coding for both the transmembrane and cytoplasmic domains. Northern blot analysis of RNA from alveolar macrophages revealed an approximate 3:1 ratio of the transmembrane form of B7-1 mRNA relative to sB7-1 mRNA. Porcine B7-1 was present on the surface of both B and T cells following stimulation with PMA/ionomycin. A histidine-tagged form of porcine sB7-1 (sB7-1-His) interacted with both CD28 and CTLA-4, and effectively blocked IL-2 production from human responder cells stimulated with PHA and either porcine or human stimulator cells. In addition, sB7-1-His inhibited human T cell proliferation in response to porcine or human peripheral blood leukocytes. This study is the first report of an alternatively spliced form of B7 that codes for a soluble protein. Furthermore, these data demonstrate that porcine B7-1 interacts with the human receptors CD28 and CTLA-4, suggesting a potential role for this molecule in pig to human xenotransplantation. Possible physiological functions for the soluble form of B7-1 are discussed.

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Materials and Methods

Abs and recombinant proteins

Various mAbs reactive with porcine cell surface markers were purchased from VMRD (Pullman, WA), including anti-CD3 (clone 8E6), anti-IgM (clone PG145A), anti-class I (clone PT85A), and anti-class II (clone MSA3). The anti-porcine B7-2 mAb was generated at Alexion Pharmaceuticals (New Haven, CT). The functionally blocking anti-CD28 mAb 9.3 was a kind gift of Dr. Carl June (Department of Molecular and Cellular Engineering, University of Pennsylvania, Philadelphia, PA), and the anti-CD59 mAb MEM43 was obtained from Biodesig International (Kennebunkport, ME). Rabbit antiserum directed against porcine B7-1 was generated by repeated s.c. immunization with sB7-1 containing six histidine peptides. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 Abbreviations used in this paper: sB7-1, soluble B7-1; hB7-1, human B7-1; PAEC, porcine aortic endothelial cell; tmB7-1, transmembrane B7-1; UTR, untranslated region.

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under the following reaction conditions: 50 mM KCl, 10 mM Tris-HCl (pH 8.3) generated by PCR. This DNA fragment was used to screen a cloned into the pCR2.1 vector using the T/A cloning system, as described cycle extension step at 72°C for 10 min. The resulting 338-bp fragment was sequenced to rule out potential errors induced during amplification.

Cells and cell lines
To purify BPL, heparinized porcine peripheral blood was obtained from adult swine (Cocalico). Human peripheral blood was collected from healthy adult volunteers by venipuncture. Blood samples from either species were diluted 1/2 with HBSS (Life Technologies, Grand Island, NY), and the nonmonoclonal fraction containing BPL was obtained by centrifugation over a Ficoll density gradient (Lymphocyte Separation Medium; Cellgro, Herndon, VA). Low density cells were collected from the interface and washed repeatedly in PBS containing 5% heat-inactivated bovine FCS (HyClone, Logan, UT). Viable cells were enumerated by trypan blue exclusion. The human T cell line, Jurkat, and the human B cell line, Raji, were obtained from American Type Culture Collection (Manassas, VA). Porcine aortic endothelial cells (PAEC) were purchased from Cell Systems (Kirkland, WA).

Cloning of porcine B7-1
Total RNA was prepared from freshly isolated porcine BPL using the acid/ guanidinium thiocyanate technique (18). Ten micrograms of total RNA were heated at 65°C for 3 min, quenched on ice, and subjected to first strand cDNA synthesis for 1 h at 37°C in the following 100 µl reaction mixture: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 10 mM dithiothreitol, 0.20 mM of each dNTP, 0.5 µg oligo(dT), and 20 U of avian myeloblastosis virus reverse transcriptase (Seikagaku, Rockville, MD). The following oligonucleotide primers synthesized at Oligos Etc. (Wilsonville, OR) were generated from regions of high homology between human and mouse B7-1 sequence: 1) 5’-TGGGGGATCCATAGAAGAC CGGAC-3’ and 2) 5’-TCAGTTTACAGCTTGCGAAA-3’. A 5-µl aliquot of the cDNA pool was used as a template in a 100 µl PCR reaction under the following reaction conditions: 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM MgCl₂, 0.1% (v/v) gelatin, 1% Triton X-100, 200 µM each dNTP, 2.5 U Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 25 pmol of each primer. PCR amplification was performed for 30 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min), followed by a 1 cycle extension step at 72°C for 10 min. The resulting 338-bp fragment was cloned into the pCR2.1 vector, as described by the manufacturer (Invitrogen, San Diego, CA) and identified by sequence analysis as a B7-1 homologue. Two gene-specific oligonucleotide primers were designed from the porcine B7-1 sequence, and a 250-bp fragment was generated by PCR. This DNA fragment was used to screen an Agt10 porcine macrophage library (a generous gift from Dr. Michael Murtaugh, Department of Veterinary Pathobiology, University of Minnesota, St. Paul, MN).

To screen the Agt10 porcine macrophage library, approximately 1×10¹⁰ plaques were isolated on nitrocellulose filters (Schleicher & Schuell, Keene, NH). Filters were denatured for 1.5 min (1.5 M NaCl and 0.5 N NaOH), neutralized for 5 min (1.5 M NaCl and 0.5 M Tris, pH 8.5), rinsed in 3× SSC, air dried, and UV cross-linked in a UV Stratalinker 2000 (Stratagene, La Jolla, CA). Filters were then prehybridized in BSA/SDS buffer (1% BSA, 7% SDS, 0.5 M sodium phosphate buffer, pH 6.8, and 1 mM EDTA) for 1 h at 60°C before addition of the porcine B7-1 fragment, previously labeled with α-³²P (NEN, Pittsburgh, PA) using the Prime-It II random primer kit (Stratagene). To a sp. act. of 1×10⁹ cpm/µg of DNA. Membranes were hybridized at 60°C overnight and subsequently washed using the following conditions: two 30-min washes with 2× SSC/0.1% SDS at room temperature, one 30-min wash with 0.5× SSC/0.1% SDS at 50°C, and one 30-min wash with 0.2× SSC/0.1% SDS at 60°C. Positive plaques present on duplicate lifts were fixed and the B7-1 DNA was rescued by PCR using primers that flanked the insertion site of the Agt10 vector (Clontech, Palo Alto, CA). After cloning the PCR fragment into pCR2.1-TOPO, both strands of the putative full-length clone were sequenced using the chain termination method. Clones derived from different PCR reactions were also sequenced to rule out potential errors induced during amplification. The human B7-1 cDNA was then cloned into the pCR2.1 vector using the T/A cloning system, as described.

The transmembrane form of porcine B7-1 (tmB7-1) was isolated by RT-PCR of freshly isolated porcine lung RNA using an oligonucleotide from the 3' end of the sB7-1 coding region as the 5' primer (GCCT TTACAGCTTGCGAAA-3') and oligo(dT) as the 3' primer. Conditions for RNA isolation and RT-PCR were otherwise identical to those described above. The two major products resulting from the RT-PCR were cloned into PCR2.1-TOPO, and inserts were sequenced for identification.

Generation of His-tagged sB7-1
sB7-1 tagged with a carboxyl-terminal histidine hexapeptide (sB7-1-His) was generated in the mammalian expression vector Apex3P (19) by PCR amplification of B7-1 cDNA. The 5' primer (GGCCTTACAGCTTGCGAAA-3') and oligo(dT) as the 3' primer. Conditions for RNA isolation and RT-PCR were otherwise identical to those described above. The two major products resulting from the RT-PCR were cloned into PCR2.1-TOPO, and inserts were sequenced for identification.

Northern blot analysis
Total RNA from porcine alveolar macrophages was kindly provided by Dr. Michael Murtaugh. Northern blot analysis was performed using the NorthernMax Kit based on the manufacturer's protocol (Ambion, Arlington Heights, IL) and a total of 10 µg of RNA per lane. Blots were hybridized with various α-²⁰P-labeled RNA probes that were generated using the MAXScript In Vitro Transcription Kit (Ambion). RNA transcripts were synthesized from B7-1 DNA fragments contained in PCR2.1-TOPO. These fragments consisted of either the complete 3' UTR of sB7-1 (soluble probe), the complete transmembrane and cytoplasmic regions of tmB7-1 (transmembrane probe), or the extracellular domain, which is common to both the soluble and transmembrane forms of B7-1 (common probe). Levels of each transcript were determined by densitometry using a Gel Doc 1000 (Bio-Rad, Hercules, CA) and NIH Image 1.61 software (downloaded from http://rsb.info.nih.gov/nih-image/download.html).

PMA/ionomycin activation of porcine BPL
Porcine PBL intended for use as stimulator cells in mixed allogeneic or xenogeneic lymphocyte cultures (see below) were resuspended in R10 medium (RPMI containing 5×10⁻³ M 2-ME, 10 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS) supplemented with 1 ng/ml PMA (Sigma, St. Louis, MO) and 400 ng/ml ionomycin (Sigma). The cells were seeded in 24-well plates at 2.5×10⁶ cells/ml in a final volume of 2 ml, and incubated for 48–72 h at 37°C in 5% CO₂ air. Mitomycin C (50 µg/ml; Sigma) was added to the cells during the last 30 min of culture. Activated cells were then harvested and washed extensively to remove residual PMA/ionomycin and/or mitomycin C. Porcine PBL used in FACScan analysis were stimulated with 20 ng/ml PMA and 200 ng/ml ionomycin for 48 h.

Immunofluorescence and flow cytometry
Activated cell populations were preincubated in PBS containing 5% goat serum. Cells were then incubated with purified IgG from rabbit immunized with sB7-1-His (rabbit anti-porcine-B7-1) or from anti-murine rabbit serum, in combination with murine mAbs directed against porcine CD3, IgM, class II, and B-7 cell surface Ags, in PBS containing 2% goat serum. Cells were washed in the same and then reacted with FITC-labeled goat anti-mouse Ig and with PE-labeled goat...
anti-rabbit Ig. The cells were again washed and analyzed for surface immuno-
fluorescence using a FACSort flow cytometer and CellQuest Soft-
ware (Becton Dickinson, Mountain View, CA). Further analysis was per-
fomed using WinMDI version 2.7 software (provided by Dr. Joseph
Trotter, University of San Diego, CA).

In experiments performed to assess the binding of sB7-1 to the surface of
Jurkat cells, sB7-1-His (2.5 μg/ml) was preincubated in HBSS contain-
ing either anti-CD28 mAb (25 μg/ml), anti-CD59 mAb (20 μg/ml), anti-
CD3 mAb (20 μg/ml), human CTLA-4Ig (20 μg/ml), or buffer alone be-
fore being added to Jurkat cells (2.5 × 10^6 cells/well) for an additional
incubation. Cells were then incubated with purified rabbit anti-porcine-
B7-1 IgG (10 μg/ml), washed in HBSS, and finally incubated in the FITC-
conjugated goat anti-rabbit secondary reagent (1/100 dilution). All incu-
Bations were performed for 30 min at 4°C. Jurkat/sB7-1-His binding was
detected by cell surface immunofluorescence and flow cytometry, as de-
scribed above.

Costimulation assays

The Jurkat T cell costimulatory assay has been described previously (21,
22). Briefly, PAEC were seeded in wells of 96-well microtiter plates at 5 ×
10^4 cells/well in R10 medium, and cells were allowed to adhere overnight
at 37°C. Jurkat T cells (1 × 10^5 cells/well) were then added to the wells
in the presence or absence of 10 μg/ml PHA (Sigma) and either serial dilu-
tions of sB7-1-His, 50 μg/ml of human CTLA-4Ig, or 100 μg/ml of por-
cine P-selectin-His. In some experiments, Raji cells (1 × 10^5 cells/well)
were substituted for PAEC, but were added to the Jurkat cells at the ini-
tiation of the experiment. The cultures were maintained at 37°C in 5% CO_2
for 24 h, at which time the culture supernatants were harvested. IL-2 was
measured in supernatants using an ELISA kit (Quantikine Human IL-2
Immunoassay; R&D Systems, Minneapolis, MN), according to the manu-
facturer’s protocol. Briefly, serial dilutions of a human rIL-2 standard (R&D
Systems) or culture supernatants were added, in triplicate, to an ELISA
well that had been previously coated with a mAb specific for human IL-2,
and plates were incubated overnight at 4°C. Unbound cytokine was re-
moved by repeated washes. Bound IL-2 was detected using a second IL-
2-specific Ab conjugated with HRP, followed by addition of a hydrogen
peroxide/chromogen substrate. The reaction was stopped by the addition
of 2 N sulfuric acid. The OD of each well was determined using a microtiter
plate reader (Bio-Rad model 3550, Hercules, CA) set to 450 nm, with
values corrected by subtraction of readings taken at 595 nm. IL-2 concen-
trations were calculated using Microplate Manager software (Bio-Rad).

Mixed lymphocyte reactions

Stimulator cells (mitomycin C-treated allogeneic human PBL or PMA/
ionomycin-activated porcine PBL; 5 × 10^6 cells/well) and responder lymph-
ocytes (human PBL; 5 × 10^5 cells/well) were combined in wells of a
96-well microtiter plate in the presence or absence of serially diluted sB7-
1-His, or of the indicated concentrations of CTLA-4Ig or porcine P-select-
in-His (final volume 0.2 ml/well). Cells were maintained in R10 medium
for 4–5 days at 37°C in 5% CO_2 in air. [3H]thymidine (1 Ci/well; NEN
DuPont, Boston, MA) was added to the cell cultures during the last 16–18
h of the incubation. The cells were harvested onto glass fiber filters with
an automated sample harvester (Wallac, Turku, Finland) and the filters
were counted in a beta liquid scintillation counter (Wallac).

Western blot analysis

Supernatants from 293 cells transiently transfected with sB7-1/Apex3P,
sB7-1-His/Apex3P, or Apex3P alone were subjected to SDS-PAGE (4–
12% gradient gels) under reducing or nonreducing conditions. Proteins
were transferred to nitrocellulose and the membrane was blocked for 1 h in
blocking solution (Tris-buffered saline containing 5% dry milk). Blots were
incubated for 1 h in fresh blocking solution containing either rabbit anti-
histidine polyclonal IgG (0.2 mg/ml) or purified anti-porcine B7-1 poly-
elonal IgG (2 mg/ml). Blots were then washed three times with Ab wash
solution (500 mM NaCl, 35 mM Tris, pH 7.4, 0.5 mM CaCl_2, 0.1% SDS,
1% Nonidet P-40, and 0.5% deoxycholic acid) before the addition of fresh
blocking solution containing HRP-conjugated goat anti-rabbit secondary
Ab (1:5000; Zymed) for 15 min. Blots were washed three times in Ab wash
solution, incubated for 1 min in ECL Western blot reagent, and
exposed to ECL Hyperfilm (both from Amersham, Arlington Heights, IL).

Results

Molecular cloning and sequence analysis of porcine B7-1

Signals generated by the interactions of CD28 and CTLA-4 with the B7
molecules have been shown to be critically involved in
mediating experimental allograft rejection (23, 24). In addition, an
important role for these molecules in cellular xenograft rejection is
suggested by data showing enhanced graft survival when
CD28-B7 interactions are inhibited (25). The demonstration of
functional CD28-B7 interactions across the species barrier in the
potentially clinically relevant porcine to human xenotransplant
model could have significant therapeutic implications, given the
demonstrated potency of the human anti-pig cellular immune
response.

A recent study indicated that porcine B7-2 is recognized by
human CD28, and that this interaction promotes activation of hu-
man T cells by porcine APCs (26). To further investigate the im-
portance of porcine B7 molecules in the human anti-pig immune
response, porcine B7-1 was cloned from a lgt10 library generated from
porcine lung macrophages. In the initial cloning attempts, only
CDNA encoding sB7-1 was obtained from the macrophage library, at a frequency of approximately one clone per 1 × 10^6
phase. This full-length clone lacked both the transmembrane and
cytoplasmic domains, sB7-1 cDNA contained 1206 bp comprised of
a 304 bp 5′ UTR, a 215 bp 3′ UTR including a processing/
polyadenylation signal, and an open reading frame that encoded
229 aa (data not shown; GenBank accession number AF203442).

The abnormally long 5′ UTR observed for sB7-1 corresponded to
that of B7-1 reported for other species (4, 27). To obtain tmB7-1,
RT-PCR was performed on porcine lung RNA using a 5′ primer
recognized from the end of the sB7-1 coding region and oli-
godT_d). A B7-1-specific band of approximately 340 bp was gen-
erated that encoded a putative transmembrane domain and a partial
cytoplasmic domain, but lacked sequence encoding the transla-
tional stop site and the 3′ UTR (data not shown; GenBank acces-
sion number AF203443).

The truncation of tmB7-1 cDNA derived from reverse-transcribed porcine lung RNA and the lack of detec-
tion of tmB7-1 in the oligo(dT)-primed porcine macrophage li-
brane suggest strong 3′ UTR secondary structure in this transcript.

The predicted amino acid sequences for sB7-1, tmB7-1, and
human B7-1 (hB7-1) were compared (Fig. 1A). Sequences were
segregated into domains based on exon boundaries identified for
hB7-1 (15). Considering that sB7-1 and tmB7-1 amino acid se-
quences were identical before the transmembrane domain, only
B7-1 is depicted before this region. Excluding the transmembrane
and cytoplasmic domains, which are highly divergent between spe-
cies, porcine B7-1 and hB7-1 shared 65% sequence identity and an
overall conservation of the Ig V-like and Ig C-like structural do-
 mains characteristic of other B7 molecules (28). The signal peptide
for sB7-1 was 29 aa in length, as determined by amino-terminal
sequencing of purified protein. Virtually all amino acid residues
that have been shown to be critical for the binding of B7-1 to both
CD28 and CTLA-4 (excluding methionine 47 and isoleucine 49)
were highly conserved (29–33). A clone containing the complete
coding region for tmB7-1 was not found, but based on sequence
comparison with various other species, the terminal amino acid is
expected to be very close to the translational stop site.

Various splice variants have been reported for the B7 molecules,
none of which encode a soluble product (13, 14, 17). To examine
the splicing mechanism that generated sB7-1, the nucleic acid and
amino acid sequences corresponding to the junction of exons 4 and
5 of hB7-1 were compared with porcine sB7-1 and tmB7-1 (Fig.
1B). Sequence identity between the alternative forms of porcine
B7-1 were identical in exon 4, but showed no homology beginning
with exon 5. A stop codon was generated in the beginning of exon
5 for sB7-1. These data suggest that sB7-1 is a splice variant lack-
ing exons coding for both the transmembrane and cytoplasmic do-
 mains and thus represent the first report of a naturally occurring
soluble form of B7-1.
To determine the relative levels of expression of sB7-1 and tmB7-1 transcripts, Northern blot analysis was performed on total RNA isolated from porcine alveolar macrophages. Reactive RNA species were detected with either a sB7-1-specific probe, a tmB7-1-specific probe, or a probe common to both sB7-1 and tmB7-1. The sB7-1-specific probe hybridized with a single species of approximately 1.3 kb (Fig. 2). The size of this message approximates the size of the sB7-1 cDNA clone isolated from the porcine macrophage library. The tmB7-1-specific probe hybridized with a transcript of approximately 3 kb, which represents the membrane form of porcine B7-1. These data indicate that both sB7-1 and tmB7-1 transcripts are well represented in porcine alveolar macrophages.

**FIGURE 1.** Sequence comparisons between porcine and human B7-1. A. Amino acid sequences of sB7-1, tmB7-1, and hB7-1 were aligned based on amino acid identity and structural similarity. Identical amino acids are denoted by asterisks, and gaps in the sequences are indicated by dashes. Assignment of structural domains is based on exon boundaries published for hB7-1 and are identified by the following abbreviations: signal peptide, SP; Ig variable-like domain, Ig V-like; Ig constant-like domain, Ig C-like; transmembrane domain, TM; cytoplasmic domain, CYT. The signal peptide is depicted by single underline. The transmembrane domain was determined using the PSORT II program (http://psort.nibb.ac.jp) and is indicated by dashed underline. Sites known to be critical for B7-1 binding to CD28 and/or CTLA-4 are shown by double underline. Translational stop sites are indicated by closed diamonds, while the closed circles at the end of the tmB7-1 sequence indicate that the stop codon was not identified in this molecule. The transmembrane and cytoplasmic domains are absent in sB7-1. B. Partial nucleic acid sequence flanking the junction site of hB7-1 exons 4 and 5 was aligned with sB7-1 and tmB7-1 based on sequence similarities. Encoded amino acid sequences for each are also shown. Protein domains corresponding to exons 4 and 5 are depicted as extracellular or transmembrane. The translational stop site for sB7-1 is indicated by an asterisk.
Most studies have indicated that the B7-1 costimulatory molecule is either undetectable or present on only a small subset of resting human B and T cells, but that it is dramatically up-regulated in these cell types upon stimulation by various means (34). To assess the cell surface expression of porcine B7-1 on various subsets of stimulated cells, porcine PBL were evaluated following stimulation with PMA/ionomycin using two-color immunofluorescence (B7-1 was detected by PE-, while other cell surface markers were detected by FITC-conjugated secondary reagents). Following stimulation with PMA/ionomycin, the majority of CD3-, class II-, and B7-2-positive cells were also B7-1 positive (Fig. 3, B, C and E, respectively). Virtually all IgM-positive cells were B7-1 positive (Fig. 3 D). These data suggest that a membrane-bound form of porcine B7-1 is abundant on the surface of both peripheral T cells and APC following cell stimulation.

sB7-1 binds human CD28 and CTLA-4

The ligands for several porcine adhesion and costimulatory molecules have been shown to be conserved across species, including humans. These include the ligands for porcine E-selectin, VCAM, and B7-2 (20, 26, 35). The fact that amino acids generally shown to be critical for the binding of B7-1 to both CD28 and CTLA-4 are conserved in porcine B7-1 (Fig. 1 A) suggests that this molecule could interact with human CD28 and CTLA-4. To confirm this prediction, purified sB7-1-His was incubated with human Jurkat cells. Jurkat cells constitutively express CD28, but do not express CTLA-4 under any culture conditions (36). sB7-1-His specifically bound to Jurkat cells (Fig. 4 A), but failed to bind a Jurkat derivative cell line that does not express CD28 (TIB 153, data not shown). The binding of sB7-1-His to CD28 was effectively blocked by an anti-CD28 mAb, further establishing the specificity of this interaction. An isotype-matched irrelevant mAb did not interfere with sB7-1-His/CD28 binding.

It has been demonstrated that both CD28 and CTLA-4 bind to very similar sites on B7-1 (29). To analyze the ability of porcine B7-1 to bind to CTLA-4, sB7-1-His was preincubated with human CTLA-4Ig before its addition to Jurkat cells. Human CTLA-4Ig effectively inhibited the binding of sB7-1-His to CD28 on these cells (Fig. 4 B). By contrast, an isotype-matched irrelevant mAb had no effect on sB7-1-His/CD28 binding. The interaction between porcine B7-1 and human CTLA-4 was also confirmed by ELISA, as plate-coated sB7-1-His bound to CTLA-4Ig in a titratable fashion (data not shown). These data demonstrate that a naturally occurring soluble form of porcine B7-1 has the ability to bind both human CD28 and CTLA-4. Furthermore, the binding of porcine B7-1 to human receptors suggests that this interaction could play a role in T cell activation during pig to human xenotransplantation.

sB7-1 inhibits IL-2 production following costimulation of human Jurkat T cells

To examine whether sB7-1 could interact functionally with human T cells, sB7-1-His was titrated into the Jurkat costimulation assay and its effect on T cell activation was evaluated by detection of IL-2 in the culture supernatants. Jurkat cells do not constitutively
Addition of sB7-1-His inhibited the IL-2 production when stimulated with PHA in the presence of either PAEC (Fig. 3A). By contrast, Jurkat cells generated high levels of IL-2 and detectable IL-2 under any of the culture conditions tested (data not shown). Since Jurkat cells do not express CTLA-4, these data suggest that sB7-1-His inhibits IL-2 production in these assays. Nonspecific toxicity of the sB7-1-His preparation due to excipient effects was excluded by evaluating similar volumes of dialysis buffer, collected during the final dialysis of the protein (data not shown). Since Jurkat cells do not express CTLA-4, these data suggest that sB7-1-His inhibits IL-2 production by binding to CD28 and blocking signaling through this molecule.

sB7-1 inhibits human allogeneic and xenogeneic MLRs

To determine the potential effect of sB7-1-His on T cell activation under conditions in which both CD28 and CTLA-4 molecules were present, MLRs were performed. Short-term primary human allogeneic and xenogeneic MLRs were established by coculturing human PBL with mitomycin C-treated human (Fig. 6A) or porcine species at approximately 80 kDa, presumably due to dimerization of increasing amounts of sB7-1-His, an irrelevant histidine-tagged protein, or CTLA-4Ig, and responder cell proliferation was assessed on day 4–5 of culture. The addition of sB7-1-His effectively inhibited human T cell proliferation in a dose-dependent fashion in response to both allogeneic and xenogeneic stimulator cells (Fig. 6, A and B, respectively). Addition of human CTLA-4Ig at concentrations of 100 μg/ml also effectively inhibited cell proliferation in both assays, while addition of porcine P-selectin-His at 100 μg/ml had no effect. These results indicate that binding of porcine sB7-1-His to CD28 and/or CTLA-4 ligands on human T cells inhibits their activation by allogeneic or xenogeneic stimulation in a concentration-dependent manner.

sB7-1 exists as both a monomer and dimer

The generation of sB7-1 through alternative splicing creates a cysteine residue before a translational termination signal (Fig. 1). Although this cysteine residue is included in the recombinantly produced protein, the addition of a histidine tag on the carboxyl terminus of sB7-1 could affect potential disulfide bond formation, and thus dimerization of the molecule. To investigate the ability of the native molecule to dimerize, sB7-1 was transiently expressed in 293 cells and compared with sB7-1-His under reducing and nonreducing gel electrophoresis, followed by Western blot analysis. Under reducing conditions, both proteins migrated as a doublet at approximately 40 kDa, with the histidine-tagged version of the protein running just above the untagged version, most likely due to the hexapeptide tag (Fig. 7A). Conversely, while sB7-1-His ran identically under nonreducing conditions, the untagged version of sB7-1 showed an additional major species at approximately 80 kDa, presumably due to dimerization of increasing amounts of sB7-1-His, an irrelevant histidine-tagged protein, or CTLA-4Ig, and responder cell proliferation was assessed on day 4–5 of culture. The addition of sB7-1-His effectively inhibited human T cell proliferation in a dose-dependent fashion in response to both allogeneic and xenogeneic stimulator cells (Fig. 6, A and B, respectively). Addition of human CTLA-4Ig at concentrations of 100 μg/ml also effectively inhibited cell proliferation in both assays, while addition of porcine P-selectin-His at 100 μg/ml had no effect. These results indicate that binding of porcine sB7-1-His to CD28 and/or CTLA-4 ligands on human T cells inhibits their activation by allogeneic or xenogeneic stimulation in a concentration-dependent manner.
of the molecule through cysteine bonding (Fig. 7C, lanes 2 and 3, respectively). As expected, Ab reactive against the histidine tag recognized sB7-1–His, but not the untagged version of the protein (Fig. 7A, lanes 2 and 3, respectively). No reactivity was observed in samples prepared from 293 cells transfected with vector alone (Fig. 7, all panels, lane 1). These results suggest that native sB7-1 may exist as both a monomer and dimer. Furthermore, preliminary studies with sB7-1 preparations containing primarily homodimer indicate that this multimer also functions to block T cell activation in an MLR (data not shown).

Discussion
The physiological significance of soluble forms of otherwise transmembrane-linked proteins is not known. However, the levels of soluble adhesion molecules and cytokine receptors, whether products of alternative splicing or enzymatic cleavage, increase during inflammation, infection, or malignancies, and the majority of these soluble molecules maintain the ability to bind their ligands (38). Furthermore, an anti-inflammatory role for soluble P-selectin has been suggested by evidence that it can inhibit both CD18-dependent neutrophil adhesion to endothelium and neutrophil superoxide release (39, 40). Potential immune modulation by a soluble cytokine receptor has also been suggested by the observation that mutations in the extracellular domain of the 55-kDa TNFR-1 in individuals with autoimmune inflammatory syndrome result in a decrease in the shedding of the functionally antagonistic soluble form of the receptor (41).

The naturally occurring soluble form of B7-1 described in the present study represents a potential regulatory component of the immune system that heretofore has not been described. A histidine-tagged version of sB7-1 effectively bound CD28 and CTLA-4 molecules and blocked T cell activation in both allogeneic and xenogeneic MLRs. B7-1 has previously been expressed as a recombinant soluble molecule by replacing the transmembrane and cytoplasmic domains with the Fc region of an Ab or with an oligohistidine tag (2, 42, 43). These recombinant soluble B7-1 molecules (rsB7-1) also maintain their ability to bind both CD28 and CTLA-4 (2, 42–44). In addition, plate-immobilized rsB7-1 effectively stimulates T cells in conjunction with a primary signal (anti-CD3 mAb) (2, 43). The ability of these recombinant molecules to block T cell activation was not tested.

Inhibition of T cell activation by sB7-1–His most likely occurs through the inhibition of CD28 binding to B7-1 and/or B7-2 on the APCs. Since it has been shown that Ag presentation in the absence of costimulation through CD28 results in T cell anergy (5, 6), it is interesting to speculate that endogenous sB7-1 may exert this same effect in vivo by blocking the binding of CD28 to its ligands. Blockade of CD28 interactions with the B7 proteins may also reduce the production of Bcl-xL, a molecule important in preventing apoptosis, as engagement of CD28 has been shown to promote the production of this survival factor (45).

In the present study, the inclusion of a carboxyl-terminal histidine hexapeptide prevented B7-1 disulfide-linked dimer formation, as an untagged version of the molecule migrated primarily as a dimer under nonreducing electrophoretic conditions, while the histidine-tagged version ran entirely as a monomer. Apparently, the highly charged histidine tag restricts disulfide bond formation between cysteine residues found at the carboxyl end of the molecule. This finding raises the question of whether sB7-1–His may be functionally different from the untagged version of the molecule. Our
preliminary studies with sB7-1 preparations containing primarily homodimer indicate that the homodimer also blocks T cell activation in an MLR (data not shown). In addition, the recent resolution of the crystal structure of human rsB7-1 indicates that this molecule undergoes a rapid monomer-dimer exchange that favors non-disulfide-bonded homodimer formation (46). These data suggest the possibility that sB7-1-His utilized in the present study may also exist as a homodimer.

The ability of sB7-1 to form a stable homodimer may have other important functional consequences. Based on crystal structure data, it has been predicted that the increase in avidity between B7-1 and CTLA-4 homodimers may serve to stabilize the B7-1/CTLA-4 signaling complex, which would facilitate the termination of T cell activation (46). It is interesting to speculate that cysteine bond formation between sB7-1 molecules would further favor the generation of stable homodimers. However, it is not known whether a sB7-1 homodimer would actively signal through CTLA-4 or alternatively, block CTLA-4 signaling by competing with B7-1 on the surface of the APC. Definitive studies addressing the physiologic function(s) of sB7-1 are currently underway.

Alternative splicing of a heteronuclear transcript can result in the generation of a soluble molecule from an otherwise membrane-linked protein. This generally occurs by removal of an exon coding for the transmembrane domain by use of an alternative downstream splice site. Examples of soluble proteins that are generated through this mechanism include LFA-3, P-selectin, and many of the cytokine receptors (38, 47). Alternatively spliced products have also been described for both B7-1 and B7-2 (13, 14), but to our knowledge, this is the first report of an abundant endogenous mRNA that lacks the transmembrane and cytoplasmic domains and encodes a functional, soluble B7 protein.

Evidence that sB7-1 reported in the present study is indeed an alternatively spliced product and not an incompletely processed mRNA with the stop codon generated from unspliced intron sequence includes the following: 1) the 1.2-kb full-length cDNA for sB7-1 corresponds in size to one of two species identified by Northern blot analysis of RNA derived from porcine macrophages, and a probe generated from sequence specific to sB7-1 (3’ UTR sequence) recognized this transcript; 2) a known processing/polyadenylation signal is found at the end of the 3’ UTR sequence of sB7-1; 3) the sB7-1 message is well represented in the porcine macrophage cDNA library; and 4) there is an absence of a splice donor site at the point that the sB7-1 cDNA sequence diverges from that of tmB7-1. In addition, using PCR analysis of porcine genomic DNA, we demonstrated that the end of the sB7-1 coding region is not juxtaposed to the downstream untranslated region, providing further support that this sequence is not unspliced intron (data not shown).

A tmB7-1-specific probe recognized a mRNA species of approximately 3 kb by Northern blot analysis, which corresponds to that reported previously for stimulated porcine lymph node cells (11). However, this study did not demonstrate the presence of the sB7-1 transcript in lymph node RNA, which may indicate its absence or low level of expression in this tissue source. Differential expression of alternatively spliced B7 products in various cell types and in response to different stimuli has been previously reported (13, 48).

Although mRNA for sB7-1 was not as abundant as the tmB7-1 transcript (3-fold less), it was well represented in the alveolar macrophage library. The complete absence of tmB7-1 cDNAs allowed the total number of clones representing sB7-1 to be determined. Of a total of approximately 3 × 10⁶ plated phage, approximately 30 sB7-1 clones were isolated (1 clone per 1 × 10⁷ phage). This frequency is indicative of a low-abundance mRNA (mRNAs oc-