Production of Recombinant Human Trimeric CD137L (4-1BBL)
CROSS-LINKING IS ESSENTIAL TO ITS T CELL CO-STIMULATION ACTIVITY*

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The interaction between 4-1BB ligand (CD137L), a member of the tumor necrosis factor superfamily, and its receptor 4-1BB provides a co-stimulatory signal for T lymphocyte proliferation and survival. However, the structure of 4-1BBL has not been thoroughly investigated, and none of the human recombinant 4-1BBL molecules available have been described as capable of co-stimulating T cells. The present work provides a model of the three-dimensional structure of the tumor necrosis factor homology domain of 4-1BBL and describes the production of a recombinant human soluble 4-1BBL whose originality lies in that it contains the whole extracellular tail preceding the tumor necrosis factor homology domain and an AviTag peptide (AviTag-4-1BBL) allowing enzymatic biotinylation and multimerization via streptavidin. We provide evidence that this chimeric protein exists as a homotrimer, whereas commercial FLAG-tagged 4-1BBL does not. This resulted in a much higher affinity for 4-1BB (1.2 nM) as compared with FLAG-4-1BBL (55.2 nM). We demonstrate that the single extracellular cysteine residue in the tail (Cys-51) could form a disulfide bond, both in our recombinant protein and in physiologically expressed 4-1BBL. The mutation of this cysteine residue exerted no effect on trimerization but increased the dissociation rate of AviTag-4-1BBL from 4-1BB. In its soluble form, AviTag-4-1BBL did not stimulate purified T cells but dramatically inhibited proliferation of peripheral blood mononuclear cells stimulated with anti-CD3 mAb. In contrast, a very significant co-stimulatory effect was observed on purified T cells once AviTag-4-1BBL was immobilized onto streptavidin beads. In addition, we show that the cross-linking of two trimeric AviTag-4-1BBL molecules was the minimum step required to elicit significant co-stimulatory activity.

4-1BBL (CD137L), a type II glycoprotein belonging to the tumor necrosis factor superfamily, was first characterized in the mouse (1) and then in humans (2). Its expression on various antigen-presenting cells such as B cells, monocytes, and splenic dendritic cells as well as on T lymphocytes is induced upon stimulation (for review see Ref. 3). 4-1BBL interacts with 4-1BB, expressed almost exclusively on T cells following T cell receptor stimulation (4), leading to T cell proliferation and cytokine production. 4-1BB ligation also promotes T cell survival and inhibits apoptosis via the induction of the anti-apoptotic molecule Bcl-XL (5, 6). Although CD28/B7 interactions play a key role in the early phases of antigen recognition, 4-1BB stimulation seems to be implicated at a later stage in the primary immune response and during antigen re-exposure (7). Moreover, a report by Melero et al. (8) highlighted the importance of 4-1BB/4-1BBL interactions in anti-tumor immunity by showing that the administration of agonistic anti-4-1BB to the mouse resulted in dramatic tumor regressions, even in weakly immunogenic tumors.

Considering the growing interest in studying 4-1BB stimulation in T cell expansion, activation, and survival, it is surprising that only two reports have described the use of recombinant 4-1BBL (9, 10) and that it was mouse 4-1BBL that significantly differed from human 4-1BBL (only 36% homology at the protein level) in both cases. In fact, most of the in vitro and in vivo studies have been performed either with a stimulatory anti-4-1BB mAb (8, 11, 12) or with cells transfected with 4-1BBL cDNA (13–15). In addition, despite different recombinant human 4-1BBLs being commercially available today (Alexis, Chemicon, Ancell), they are poorly characterized from a biochemical point of view, and none of them are described as being able to co-stimulate T lymphocytes. As a result, little evidence has been available to date in the literature concerning the structure of 4-1BBL and its organization necessary to achieve T cell co-stimulation. For this reason, we set out to produce a soluble form of human 4-1BBL to determine its structure, its binding characteristics toward 4-1BB and to evaluate its functional activity.

MATERIALS AND METHODS

Sequence Alignment and Molecular Modeling—Sequences were obtained from the Swiss-Prot data base. Sequence alignment of selected templates was firstly deduced from a pairwise structure alignment automatically generated by the CE (16) and FSSP (17) programs. The 4-1BBL sequence was then manually aligned with the template multi-alignment according to conserved amino acids and predicted β-strand structure. Homology modeling was performed with the Modeler module of InsightII (18) using the x-ray crystallographic structure of mouse TNFa, mouse RANKL, human CD40L, and human APO2L (Protein Data Bank codes 2TNF, 1IQA, 1ALY and 1DG6 respectively). Loop refinement of selected models was performed with the refined loop Modeler program. An evaluation of the generated models was performed using the Verify-3D (19), ProsaII (20), and Procheck (21) programs. Energy minimization was then carried out with the CHARMM module of Insight and CHARMM forcefield (22) in a four-step procedure (1, all atoms fixed except hydrogen; 2, backbone fixed; 3, backbone of strand structure fixed; 4, all atoms free) using the 100 steepest descent steps followed by conjugate gradient steps, until a convergence gradient of 0.001 was attained. The energy scores provided by ProsaII and the global compatibility score given by Verify-3D were in accordance with those of the templates. Furthermore, the stereochemical quality of the selected predicted model, as evaluated by Procheck, compares favorably with the template. The Ramachandran plot showed 97.7% residues in allowed regions. The protein molecular surface of the selected model was defined using a solvent probe radius of 1.4 Å and continuum elec-

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Soluble Human 4-1BBL Is Co-stimulatory Only When Cross-linked

Production of AviTag-4-1BBL as Inclusion Bodies in Bacteria—The cDNA encoding the whole extracellular domain of 4-1BBL was obtained by reverse transcription PCR amplification of T2 hybridoma RNA prepared using TRIzol® (Invitrogen). Single-stranded cDNA was synthesized from 2 µg of total RNA using poly(dT) primers and M-MLV RT (200 UI, Invitrogen). The extracytoplasmic domain of 4-1BBL was amplified by PCR using the following primers: 5'-CGG-GATCCCTCGCCTGGGGCC-3' and 5'-GCTCTAGATTATTCGACCTCGGTGA-3'. The AviTag sequence (AviTag) TCCGGCCTGAACGACATCTTCGAGGCT-GATCCCTCGCCTGCCCCTGGGCC-3 was inserted into the pET24a production vector (Invitrogen). AviTag-4-1BBL was synthesized from 2 µg of total RNA using poly(dT) primers and M-MLV RT (200 UI, Invitrogen). The extracytoplasmic domain of 4-1BBL was amplified by PCR using the following primers: 5'-CGG-GATCCCTCGCCTGGGGCC-3' and 5'-GCTCTAGATTATTCGACCTCGGTGA-3'. The AviTag sequence (AviTag) TCCGGCCTGAACGACATCTTCGAGGCT-GATCCCTCGCCTGCCCCTGGGCC-3 was inserted into the pET24a production vector (Invitrogen). AviTag-4-1BBL was produced as inclusion bodies in Escherichia coli BL21(DE3) strain. The AviTag-4-1BBL protein (10 mg) was allowed to refold in 500 ml of folding buffer (0.4 M arginine, 100 mM Tris, pH 8, 2 mM EDTA, pH 8, 5 mM GSH, 0.5 mM GSSG, 0.005% Tween 20, and a protease inhibitor mixture (Complete, Roche Diagnostics) slowly stirred for 72 h at 4 °C under slow agitation, before concentration on a 10-kDa cut-off membrane (Millipore). Biotinylation was performed using biotin protein ligase (AviTag) in compliance with the manufacturer's instructions. After removal of free biotin on HisPrep desalting, AviTag-4-1BBL was purified by gel filtration on Superdex 200 HR 16/60 (Amersham Pharmacia Biotech). The mutation of AviTag-4-1BBL C51S was performed from T2 cells using Triton X-114 as previously described (26). Briefly, 60 × 10^6 T2 cells were lysed with 1 ml of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-114 (Sigma) and a protease inhibitor mixture (Complete, Boehringer) for 30 min on ice. The lysate was centrifuged at 9000 × g for 10 min to remove cell debris and nuclei, and overlaid on an ice-cold sucrose cushion (6% w/v sucrose, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.06% Triton X-114). A clumbing of the solution occurred after a 3-min incubation at 30 °C. The tube was then centrifuged for 3 min at 300 × g at room temperature to recover the detergent phase as an oily droplet from the bottom of the tube. The detergent phase enriched in membrane proteins was precipitated by MEOH/CHCl_3 (1:15, Santa Cruz Biotechnology). Revelation was performed by MEOH/CHCl_3, 4:1 (v/v) before loading onto a 12% SDS-PAGE and immunoblotting as described above.

Surface Plasmon Resonance Analyses—Binding experiments of the different recombinant 4-1BBL proteins to a recombinant human 4-1BB-Fc chimera (R&D Systems) were performed with a BIAcore 2000 optical biosensor (BIACore AB, Uppsala, Sweden). The human 4-1BB/Fc protein was covalently coupled to a carboxymethyl dextran flow cell (CM5 BIACore) as recommended by the manufacturer. The level of immobilization was set at 500 resonance units. The binding of purified mutant and native 4-1BBL was assayed at 25 °C with concentrations ranging from 0.725 to 72.46 nM at a flow rate of 40 µl/min in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20). Association was monitored for 5 min before initiating the dissociation phase for another 10 min with HBS-EP buffer. The flow cells were regenerated by a 1-min pulse with 10 mM glycine-HCL at pH 1.8. The resulting sensograms were analyzed using the BIA Evaluation software 3.2 (BIACore AB).

Multimerization of the Different Recombinant 4-1BBLs—AviTag-4-1BBL (wild type (WT) and C51S) was coated onto M280 streptavidin magnetic beads by incubating decreasing concentrations of biotinylated proteins (ranging from 10 µg/ml to 50 ng/ml) with 67.10^7 M280 magnetic microbeads (DynaBeads) for 2 h at room temperature under continuous agitation. The FLAG-4-1BBL was coated in the same conditions using M280 tosylactivated magnetic beads covalently linked to an anti-FLAG mAb (clone M2, Sigma). The saturation of the beads was checked by flow cytometry using either a mouse monoclonal PE-conjugated anti-4-1BBL (clone C65-485, BD Biosciences) or a goat anti-4-1BBL polyclonal Ab followed by an adsorbed fluorescein isothiocyanate–conjugated donkey anti-goat Ab as a secondary reagent (Serotec). Saturation, estimated by the fluorescence plateau, was obtained with 0.5 pg/bead of AviTag-4-1BBL and 2 pg/bead of FLAG-4-1BBL.

Tetramerization with streptavidin is usually attained by mixing streptavidin (Sigma) and AviTag-4-1BBL at a molecular ratio of 1:4 for 1 h at room temperature. To obtain a mixture of the different multimers...
Soluble Human 4-1BBL Is Co-stimulatory Only When Cross-linked

(tetramers, trimers, dimers, and monomers) AviTag-4-1BBL was incubated with a molar excess of streptavidin, and the different forms were then separated by gel filtration.

Cell Isolation and Functional Assays—Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation and resuspended in RPMI medium (Sigma) supplemented with 1% l-glutamine and 8% human serum. Purified T cells were obtained from fresh blood by negative selection using a RosetteSep isolation kit (StemCell Technologies). The purity of the T lymphocyte preparation was checked by flow cytometry with a fluorescein isothiocyanate-conjugated anti-CD3 mAb (clone UCHT1, BD Biosciences) immobilized on M280 streptavidin magnetic beads. OKT3 (Orthoclone) immobilized in flat bottom 96-well plates was stimulated for 72 h with biotinylated anti-CD3 mAb (clone UCHT1, BD Biosciences) immobilized on M280 streptavidin magnetic beads. OKT3 (Orthoclone) immobilized in flat bottom 96-well plates was used to stimulate PBMC (10^5/well) overnight at 4 °C in phosphate-buffered saline instead of anti-CD3-coated beads, because macrophages tended to engulf the beads. All 4-1BBL ligands were added after 6 h of CD3 stimulation to allow the induction of 4-1BB expression on T lymphocytes. Proliferation was estimated by measuring the incorporation of tritiated thymidine in quadruplicate samples during the last 16 h of culture.

The binding of the different recombinant 4-1BBLs to CD3-activated or resting T lymphocytes was evaluated after 48 h. Staining was performed at room temperature for 1 h using either PE-conjugated anti-4-1BB mAb (clone 4B4-1, BD Biosciences), AviTag-4-1BBL at 10 μg/ml revealed by PE-conjugated streptavidin at 0.5 μg/ml or FLAG-4-1BBL at 10 μg/ml revealed with a combination of mouse anti-FLAG mAb (10 μg/ml) and a fluorescein isothiocyanate-conjugated goat anti-mouse Fab′2 Ab (10 μg/ml). Staining of 4-1BBL on the T2 hybridoma was performed using PE-conjugated anti-4-1BBL (clone C65-485) for 45 min at 4 °C. Flow cytometry was performed on a FacScan (BD Biosciences) and analyzed with the CellQuest Pro software (BD Biosciences).

Statistical Analysis—Results are expressed as mean ± S.E. Results were analyzed by analysis of variance followed by either Tukey-Kramer or Dunnnett post-tests. Post-tests were only performed when the analysis of variance test showed a significant difference (p < 0.05) between groups.

RESULTS

Modeling of the Three-dimensional Structure of 4-1BBL—4-1BBL belongs to the TNF superfamily and, as such, is presumed to be trimeric like the other members of the family (27), although no information on its three-dimensional structure has yet been provided. We thus decided to generate a model of 4-1BBL structure. We firstly performed a sequence alignment of the TNF-homology domain (THD) of human 4-1BBL with that of mouse TNF, mouse RANKL, human CD40L and human APO2L (Fig. 1A). The THD of 4-1BBL displayed a 17.6, 21.6, 17.8, and 19.5% sequence homology with TNF, RANKL, CD40L, and APO2L, respectively. With this alignment and based on x-ray structures of mouse TNF, mouse RANKL, human CD40L, and human APO2L, a model of the THD of human 4-1BBL was generated (see “Materials and Methods”). A ribbon representation of the final model is given in Fig. 1B, showing two stacked β-pleated sheets each made up of five anti-parallel β-strands that adopt the classical “jelly-roll” topology found in proteins belonging to the TNF family (27). The inner sheet (strands A, A′, H, C, and F) may be involved in trimer contacts (Fig. 1B, left) and the outer sheet (strands B, B′, D, E, and G) may be exposed to the solvent (27). An analysis of hydrophobicity revealed a large hydrophobic area containing residues constituting the sheet potentially involved in trimer formation: Phe-7 and Val-11 (strand A), Val-149, Phe-153, Val-155, and Ile-159 (strand H), Val-55 and Phe-59 (strand C), Phe-112, Phe-114, Leu-118, and Leu-119 (strand F) (Fig. 1B, middle). In addition, charged residues such as Arg-65, Glu-63, and Asp-99 located close to the hydrophobic area may also contribute to trimer formation (Fig. 1B, right). A previous crystallographic analysis of the LTα/TNFRI complex showed that loops of the trimeric ligand are particularly involved in contact with the receptor, especially loops AA′, DE, EF, and CD (28). In our model, the low number of conserved residues found in those loops of 4-1BBL and the high flexibility of these regions support their implication in defining the specificity of ligand-receptor interaction.

The Extracellular Domain of 4-1BBL Forms a Trimer Containing a Disulfide Bond—To produce recombinant soluble 4-1BBL, we engineered a cDNA construct coding for the whole extracellular domain of human 4-1BBL coupled to a biotin tag, the AviTag. This construct included the 43-amino-acid long tail spanning from the membrane to the THD and thus differed from a commercially available FLAG-tagged 4-1BBL (Alexis) (Fig. 1C). It should be noted that this tail contains a cysteine residue close to the membrane.

We first produced the AviTag-4-1BBL protein with a theoretical mass of 23 kDa as inclusion bodies in E. coli. The purity of inclusion bodies exceeded 95%, as evaluated by SDS-PAGE and Coomassie staining (Fig. 2A). After refolding and concentration, gel filtration analysis revealed two major peaks, Peaks 1 and 2, with a molecular mass of around 140 and 70 kDa, respectively (Fig. 2B). Peak 2 was compatible with a trimeric form of AviTag-4-1BBL. SDS-PAGE electrophoresis followed by Western blotting of Peak 2 showed that the trimeric form dissociated into a monomer and a dimer. The homodimer contained a disulfide bond as demonstrated by its disappearance under reducing conditions (Fig. 3A). An analysis of Peak 1 by Western blot under reducing and non-reducing conditions showed an identical profile (not shown), suggesting that it corresponded to a multimeric complex of AviTag-4-1BBL linked by disulfide bonds. This form was discarded, as it was considered to be wrongly folded, because it turned out to be totally inactive in the functional assays described later. As expected, FLAG-4-1BBL appeared only as a monomer on SDS-PAGE because it lacks the cysteine-containing region (Fig. 3A).

We next sought to determine whether the disulfide bond present in the trimeric form of AviTag-4-1BBL could have been artificially generated during the refolding of bacterial inclusion bodies. For this purpose, soluble AviTag-4-1BBL was produced in the Drosophila S2 expression system. In this system, the expressed protein is naturally processed inside the cell and then excreted into the culture medium. The analysis of AviTag-4-1BBL produced in S2 supernatants revealed a similar profile as that obtained with refolded inclusion bodies (Fig. 3B). This suggested that the disulfide bond was naturally formed in eucaryotic cells during AviTag-4-1BBL processing and export. To ascertain whether the disulfide bond was actually present in physiologically expressed 4-1BBL, Western blot analyses were performed on Triton X-114 extracts from T2 hybridoma cells that constitutively express 4-1BBL (Fig. 3C). A similar profile was obtained, with a dimeric form that could be reduced by 2-mercaptoethanol (Fig. 3D), thus pointing to the physiological nature of the disulfide bond.

Finally, we performed cross-linking experiments with BS3 on refolded AviTag-4-1BBL and FLAG-4-1BBL to visualize trimers by SDS-PAGE. Cross-linking of AviTag-4-1BBL with BS3 resulted in the appearance of bands of higher molecular mass, of which one could correspond to a trimer (around 70 kDa) and another (around 140 kDa) may represent dimers of trimers (Fig. 3E). This observation further demonstrated that
AviTag-4-1BBL refolded as a trimer. In marked contrast, cross-linking of FLAG-4-1BBL revealed no trimeric forms but only a band compatible with dimers and a strong band corresponding to large aggregates that did not penetrate into the gel (Fig. 3F). Considering that the dimeric forms probably originated from the dissociation by SDS of pre-existing and partially cross-linked trimers, our data suggest that only a very small fraction of FLAG-4-1BBL was in trimeric form (and thus undetectable as such after BS3 cross-linking), whereas most of it was monomeric.

The Disulfide Bond Stabilizes Trimeric AviTag-4-1BBL.—To investigate the role of the disulfide bond in 4-1BBL structure and function, we produced a mutated AviTag-4-1BBL where cysteine was replaced by serine (C51S). Because the cysteine residue is located well outside the THD of 4-1BBL, it was anticipated that the absence of the disulfide bond would not affect trimerization but may result in a less stable trimer. As a matter of fact, C51S AviTag-4-1BBL behaved as a trimer under gel filtration after refolding and the higher molecular weight form (Peak 1)
previously seen with the WT AviTag-4-1BBL could not be detected (Fig. 4A). Western blot analysis after SDS-PAGE showed that under non-reducing conditions, the trimer dissociated into monomers only with no dimers being present, as expected (Fig. 4B). Cross-linking with BS3 on the other hand revealed both dimeric and trimeric forms of C51S AviTag-4-1BBL (Fig. 4C). It was therefore concluded that the disulfide bond is not required for AviTag-4-1BBL refolding as a trimer.

Using surface plasmon resonance, we proceeded to determine the kinetic constants of binding of native or mutated AviTag-4-1BBL and FLAG-4-1BBL to immobilized 4-1BB-Fc. The sensorgrams obtained with a range of concentrations (0.1–5 μg/ml) of each of the three proteins are shown in Fig. 5. The first striking feature observed was that FLAG-4-1BBL displayed a much lower binding affinity to 4-1BB than native or mutated AviTag-4-1BBL (K_d of 55.2 nM for FLAG-4-1BBL versus 1.2 nM for WT AviTag-4-1BBL and 2.3 nM for its C51S variant). This lower affinity mainly resulted from a 30-fold lower association rate (k_on) for FLAG-4-1BBL as compared with WT AviTag-4-1BBL (6.7 × 10^5 versus 2.1 × 10^7 M^-1 s^-1, respectively), whereas the dissociation
constants ($k_{\text{off}}$) were comparable ($2.6 \times 10^{-4} \text{ s}^{-1}$ for WT AviTag-4-1BBL versus $3.7 \times 10^{-4} \text{ s}^{-1}$ for FLAG-4-1BBL). Given that unlike the dissociation constant, the association rate depends on the concentration of ligand, the most likely interpretation of the data is that only a small fraction of FLAG-4-1BBL had the proper trimeric conformation.

A comparison between WT and C51S AviTag-4-1BBL revealed that both proteins had very similar association rates ($2.1 \times 10^5$ versus $2.3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, respectively), which is consistent with our hypothesis that the disulfide bond bears no influence on the trimeric conformation. In contrast, the proteins differed in terms of dissociation, with C51S AviTag-4-1BBL dissociating twice as fast as WT AviTag-4-1BBL ($5.2 \times 10^{-4} \text{ s}^{-1}$ versus $2.6 \times 10^{-4} \text{ s}^{-1}$, respectively) (Fig. 5). This suggests that the presence of the disulfide bond stabilizes the trimeric form of WT AviTag-4-1BBL.

**Trimeric 4-1BBL Co-stimulates T Lymphocytes Only When Cross-linked**—To investigate the functional properties of AviTag and FLAG-4-1BBL, we firstly checked their ability to recognize 4-1BB on CD3-stimulated T lymphocytes. Flow cytometry analyses revealed that biotinylated AviTag-4-1BBL stained a similar percentage of 4-1BB positive activated T cells (60%) as the anti-4-1BB mAb used as a positive control (64%), despite having a lower mean fluorescence intensity (Fig. 6). In contrast, staining with FLAG-4-1BBL was too low to conclusively identify all positive cells.

We then tested the ability of the two proteins in soluble form to co-stimulate the proliferation of either PBMC or purified T cells activated through immobilized anti-CD3 mAb. The major difference between these two experimental designs is that immobilized anti-CD3 mAb alone is sufficient to elicit a strong proliferation of PBMC because
Soluble Human 4-1BBL Is Co-stimulatory Only When Cross-linked

Because AviTag-4-1BBL showed no co-stimulatory activity when used in soluble form, it was decided to test its effect on T cell proliferation following immobilization on a matrix. For this purpose, streptavidin beads were coated with biotinylated AviTag-4-1BBL (either WT or C51S) or anti-FLAG mAb-coupled beads with FLAG-4-1BBL, and saturation of the beads was controlled by immunofluorescence with an anti-4-1BB mAb. Interestingly, similar intensities of staining could be observed with beads coated with WT or C51S AviTag-4-1BBL, but no staining was observed with beads coated with FLAG-4-1BBL (Fig. 8A).

The use of a polyclonal anti-4-1BB antibody allowed us to ascertain whether FLAG-4-1BBL was indeed coated onto the beads. A test was then performed on these beads coated with different forms of 4-1BBL to assess their ability to co-stimulate proliferation of purified T cells. As shown in Fig. 8B, the addition of beads coated with WT or C51S AviTag-4-1BBL resulted in a very significant increase in proliferation of CD3-stimulated T cells (4793 ± 426 cpm for CD3 alone versus 14335 ± 1239 cpm for CD3 + WT AviTag-4-1BBL, n = 10, p < 0.001), whereas no stimulatory effect was observed with FLAG-4-1BBL-coated beads. Similar results were obtained with beads coated with WT AviTag-4-1BBL produced in S2 insect cells (data not shown). This demonstrated that, once AviTag-4-1BBL was cross-linked on beads, it could stimulate T cell proliferation, which was not the case with FLAG-4-1BBL that remained non-stimulatory after cross-linking. In addition, it should be noted that no significant difference in stimulatory activities were seen between beads coated with WT or C51S AviTag-4-1BBL. This suggests either that cross-linking stabilized trimeric C51S AviTag-4-1BBL or that the differences in activity were overwhelmed by the amount of stimulatory signals.

We finally performed cross-linking of WT AviTag-4-1BBL with a molar excess of streptavidin to obtain all multimeric forms, from tetramers to monomers (Fig. 9A). Despite no clear cut separation of the peaks by gel filtration, narrow fractions corresponding to each peak were tested on CD3-stimulated T lymphocytes. It was observed that high complexes (tetramers and trimers) co-stimulated proliferation very efficiently and that dimers were slightly less active, whereas monomers were inactive (Fig. 9B). This confirmed the critical requirement of cross-linking to obtain a co-stimulatory effect of AviTag-4-1BBL on T cell proliferation.

DISCUSSION

We report the production, and biochemical and functional properties of a soluble form of the human 4-1BBL extracellular domain coupled to a biotinylation peptide, the AviTag. This protein was designed to include not only the THD of 4-1BBL but also the 43-amino-acid long tail. A model of the three-dimensional structure of the THD was generated that is compatible with a trimeric organization of 4-1BBL. We provide evidence by gel filtration analysis and cross-linking experiments that our chimeric protein AviTag-4-1BBL, whether produced in bacteria or in insect cells, could indeed refold as a trimer. In contrast, our data suggest that the commercial FLAG-4-1BBL, produced in HEK293 cells, was mainly present as a monomer in solution because no trimeric form and only a weak dimeric form could be detected after cross-linking with BS3. The hypothesis that FLAG-4-1BBL has a different conformation to AviTag-4-1BBL is further supported by several findings. First, our binding experiments showed that FLAG-4-1BBL displayed a much lower affinity for 4-1BB than AviTag-4-1BBL. Second, our staining experiments demonstrated that a monoclonal antibody against cell surface-expressed 4-1BBL could recognize AviTag-4-1BBL-coated beads but not FLAG-4-1BBL-coated beads (although we cannot rule out the possibility that this antibody recognized an epitope in the tail of 4-1BBL, a tail that is absent in FLAG-4-1BBL). Finally, we observed that monomeric FLAG-4-1BBL was soluble in aqueous solution, whereas monomeric AviTag-4-1BBL was not. An unsuccessful attempt was made to...
purify soluble monomeric AviTag-4-1BBL, in particular by denaturing trimers with heat or mild detergents, but no monomers were ever recovered due to aggregation (not shown). We surmise that the FLAG peptide, which is very hydrophilic, may actually hinder the trimerization of FLAG-4-1BBL while contributing to the solubility of the monomeric FLAG-4-1BBL. In support of such a supposition, a recent publication reported that short tags can have a major impact on protein conformation and crystallization (29). In our construct, the AviTag was placed at the end of the 43-amino-acid long tail, a position that apparently prevented the tag from interfering with the trimerization process.

FIGURE 7. Effect of soluble WT AviTag-4-1BBL, C51S AviTag-4-1BBL, and FLAG-4-1BBL on CD3 stimulation. A, PBMC proliferation (10^6/well) induced by immobilized anti-CD3 mAb was dose dependently inhibited by soluble WT AviTag-4-1BBL (white circles), C51S AviTag-4-1BBL (black circles), and FLAG-4-1BBL (white diamonds). Each point represents the mean inhibition ± S.E. of five distinct experiments. The mean incorporation of PBMC with anti-CD3 alone was 109639 cpm. **, p < 0.01; *, p < 0.05 when compared with CD3 alone. B, addition of soluble WT AviTag-4-1BBL or soluble FLAG-4-1BBL had no effect on the proliferation of purified T lymphocytes (10^6/well) stimulated with anti-CD3 coated beads. Shown is mean ± S.E. of three independent experiments.

FIGURE 8. A, staining of streptavidin beads coated with WT (left panels), C51S (center panels), or anti-FLAG beads with FLAG-4-1BBL (right panels) with a monoclonal mouse anti-4-1BBL Ab (top panels) or a polyclonal goat anti-4-1BBL Ab (bottom panels). Dotted lines represent control staining of uncoated beads. Mean fluorescence of stained beads is indicated in each panel. Monoclonal anti-4-1BBL did not recognize FLAG-4-1BBL. The high background staining of anti-FLAG beads alone was because of partial binding of the secondary anti-goat antibody to mouse anti-FLAG mAb. B, effect of immobilized AviTag-4-1BBLs and FLAG-4-1BBL on T cell proliferation. Beads (1.3 ± 10^5/well) coated with WT (n = 10 independent experiments) or C51S (n = 3) AviTag-4-1BBL enhanced T cell proliferation but not FLAG-4-1BBL-coated beads (n = 3). Results are expressed as mean ± S.E. ***, p < 0.001 compared with CD3 alone.

FIGURE 9. A, 500 gel filtration analysis of oligomers of WT AviTag-4-1BBL. Trimeric AviTag-4-1BBL was mixed with a molar excess of streptavidin to obtain all degrees of multimerization. Estimated molecular weight based on elution volume are indicated over each peak. The theoretical molecular weight of the different oligomers are mentioned in the inset. B, multimeric complexes (tetrameric, trimeric, and dimeric) efficiently co-stimulated purified T lymphocyte proliferation. A narrow fraction of each peak represented in A was added to purified T lymphocytes activated for 6 h with biotinylated anti-CD3 mAb coated beads. Results are expressed as mean ± S.E. of three experiments. **, p < 0.01 compared with CD3 alone.
region could form an interchain disulfide bond, and we provide evidence that this disulfide bond was naturally present in 4-1BBL expressed on T2 cells. Using BJACore analysis it is demonstrated that the abrogation of the disulfide bond by mutation of the cysteine resulted in a 2-fold higher dissociation rate of the AviTag-4-1BBL-4-1BB complex. The absence of the disulfide bond also resulted in a diminished ability of the mutant AviTag-4-1BBL to inhibit 4-1BBL-4-1BB interactions during CD3-induced proliferation of PBMC. This suggests that the disulfide bond is important for the stabilization of the trimeric form of AviTag-4-1BBL and that it may also play a role in stabilizing physiologically expressed 4-1BBL. Moreover, the presence of a single disulfide bond within trimeric 4-1BB leaves one cysteine free to establish an additional disulfide bond with an adjacent 4-1BBL trimer, leading to a higher degree of oligomerization of 4-1BBL at the cell surface. We did detect higher molecular weight forms after refolding of 4-1BBL (Fig. 2) and BS3 cross-linking (Fig. 3E) that may represent dimers of trimers, but their presence at the cell surface needs to be confirmed. In our experience these higher molecular weight forms were non-stimulatory (not shown) but this may have been because of an incorrect conformation of these forms in solution. In line with these observations, it would be useful to investigate whether an interchain disulfide bond is present in other TNF ligand family members such as CD40L, Apo-2L, or RANKL that also have cysteine residues in their tail region.

Most of the TNF ligand family members are synthesized as membrane-bound proteins, but soluble forms can be generated by limited proteolysis. Although some of the proteases involved in this process have been identified, such as ADAM proteases for TNF and RANKL (30, 31), matrilysin for FasL (32) or furins for BAFF, APRIL, TWEAK, have been identified, such as ADAM proteases for TNF and RANKL have cysteine residues in their tail region. This has been confirmed, for example, the TNF ligand family members. The biological activity of recombinant soluble 4-1BBL has already been suggested in the mouse (9). We therefore rule out the existence of the trimeric form to activate T cells. In fact, we demonstrated that AviTag-4-1BBL trimers were sufficient to trigger co-stimulatory signals, although a higher degree of oligomerization led to enhanced co-stimulation (Fig. 9). These data tally with those previously reported with FasL and CD40L showing that two trimeric FasL or two trimeric CD40L were sufficient to trigger apoptosis or B cell proliferation, respectively, whereas a higher degree of multimerization further enhanced CD40L stimulation (but not FasL) (42).

In conclusion, we have produced a soluble human 4-1BBL that should be a versatile and useful tool to study 4-1BBL-4-1BB interactions in T cell activation, because it contains the proper trimeric conformation, has a high affinity for 4-1BB, and can be used either to mimic or block these interactions.

REFERENCES

1. Goodwin, R. G., Din, W. S., Davis-Smith, T., Anderson, D. M., Gimpel, S. D., Sato, T. A., Maliszewski, C. R., Bannan, C. I., Copeland, N. G., Jenkins, N. A., Farragh, T., Armitage, R. J., Fanslow, W. C., and Smith, C. A. (1993) Eur. J. Immunol. 23, 2631–2641
2. Alderson, M. R., Smith, C. A., Tough, T. W., Davis-Smith, T., Armitage, R. J., Falk, B., Roux, E., Baker, E., Sutherland, G. R., and Din, W. S. (1994) Eur. J. Immunol. 24, 2219–2227
3. Vinay, D. S., and Kwon, B. S. (1998) Semin. Immunol. 10, 481–489
4. Kwon, B. S., and Weissman, S. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1963–1967
5. Lee, H. W., Park, S. J., Choi, B. K., Kim, H. H., Nam, K. O., and Kwon, B. S. (2002) J. Immunol. 169, 4882–4888
6. Starck, L., Scholz, C., Donker, R., and Daniel, P. T. (2005) Eur. J. Immunol. 35, 1257–1266
7. Bertram, E. M., Dawicki, W., Sedgmen, B., Bramson, J. L., Lynch, D. H., and Watts, T. H. (2004) Immunity 17, 981–988
8. Melero, I., Shuford, W. W., Newby, S. A., Arruffo, A., Ledbetter, J. A., Hellstrom, K. E., Mittler, R. S., and Chen, L. (1999) Nat. Med. 2003, 203, 682–685
9. S Sousi, K., Lee, S. Y., Cannons, I., Yeh, W. C., Santana, A., Goldstein, M. D., Bangia, N., DeBenedette, M. A., Mak, T. W., Choi, Y., and Watts, T. H. (1998) J. Exp. Med. 187, 1849–1862
10. Cannons, J. L., Lau, P., Gummman, B., DeBenedette, M. A., Yagita, H., Okumura, K., and Watts, T. H. (2001) J. Immunol. 167, 1313–1324
11. Hurtado, J. C., Kim, Y. J., and Kwon, B. S. (1997) J. Immunol. 158, 2600–2609
12. Shuford, W. W., Klussman, K., Tschichold, D. D., Loo, D. T., Chalupny, J., Siadak, A. W., Brown, T. J., Emswiler, J., Raecho, H., Larsen, C. P., Pearson, T. C., Ledbetter, J. A., Arruffo, A., and Mittler, R. S. (1997) J. Exp. Med. 186, 47–55
13. Gramaglia, I., Cooper, D., Mine, K. T., Kwon, B. S., and Croft, M. (2000) Eur. J. Immunol. 30, 392–402
14. Wen, T., Ruckzynski, J., and Watts, T. H. (2002) J. Immunol. 169, 4897–4906
15. Maas, M. V., Thomas, A. K., Leonard, D. G., Allman, D., Addya, K., Schlienger, K., Riley, J. L., and June, C. H. (2002) Nat. Biotechnol. 20, 143–148
16. Shindyalov, I. N., and Bourne, P. E. (1998) Protein Eng. 11, 739–747
17. Holm, L., and Sander, C. (1993) J. Mol. Biol. 233, 123–138
18. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
19. Eisenberg, D., Lathy, R., and Bowie, J. U. (1997) Methods Enzymol. 277, 396–404
20. Sipp, M. J. (1993) Proteins 17, 355–362
21. Laskowski, R. A., Kohn, B. S., and Vriend, G. (1996) J. Mol. Biol. 258, 505–518
22. Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., and Karplus, M. (1983) J. Comp. Chem. 4, 187–207
23. Honig, B., and Nicholls, A. (1995) Science 268, 1144–1149
24. Gilson, M. K., and Honig, B. (1988) Proteins 4, 7–18
25. Engelmann, D. M., and Steitz, T. A. (1981) Cell 23, 411–422
26. Bode, W. S. (1981) J. Biol. Chem. 256, 1604–1607
27. Bodmer, J. L., Schneider, P., and Tschopp, J. (2002) Trends Biochem. Sci. 27, 19–26
28. Bode, W. S., D’Arcy, A., Jones, W., Gentz, R., Schoenfeld, H. J., Broger, C., Locht, P., and Luescher, H. (1993) Cell 73, 451–455
29. Laskowski, R. A., Kohn, B. S., and Vriend, G. (1996) J. Mol. Biol. 258, 505–518
30. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Sririnivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fizter, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and...
Cerretti, D. P. (1997) *Nature* **385**, 729–733
31. Lum, L., Wong, B. R., Josien, R., Becherer, J. D., Erdjument-Bromage, H., Schlondorff, J., Tempst, P., Choi, Y., and Blobel, C. P. (1999) *J. Biol. Chem.* **274**, 13613–13618
32. Powell, W. C., Fingleton, B., Wilson, C. L., Boothby, M., and Matrisian, L. M. (1999) *Curr. Biol.* **9**, 1441–1447
33. Chen, Y., Molloy, S. S., Thomas, L., Gambee, J., Bachinger, H. P., Ferguson, B., Zonana, J., Thomas, G., and Morris, N. P. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 7218–7223
34. Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., Ambroso, C., Lawton, P., Bixler, S., Acha-Orbea, H., Valmori, D., Romero, P., Werner-Fafre, C., Zubler, R. H., Browning, J. L., and Tschopp, J. (1999) *J. Exp. Med.* **189**, 1747–1756
35. Smith, R. A., and Baglioni, C. (1987) *J. Biol. Chem.* **262**, 6951–6954
36. Warzocha, K., Bienvenu, J., Coiffier, B., and Salles, G. (1995) *Eur. Cytokine Netw.* **6**, 83–96
37. Tanaka, M., Suda, T., Takahashi, T., and Nagata, S. (1995) *EMBO J.* **14**, 1129–1135
38. Martinez-Lorenzo, M. J., Alava, M. A., Anel, A., Pineiro, A., and Naval, J. (1996) *Immunology* **89**, 511–517
39. Suda, T., Hashimoto, H., Tanaka, M., Ochi, T., and Nagata, S. (1997) *J. Exp. Med.* **186**, 2045–2050
40. Schneider, P., Holler, N., Bodmer, J. L., Hahne, M., Frei, K., Fontana, A., and Tschopp, J. (1998) *J. Exp. Med.* **187**, 1205–1213
41. Mazzei, G. J., Edgerton, M. D., Losberger, C., Lecoanet-Henchoz, S., Graber, P., Durandy, A., Gauchat, J. F., Bernard, A., Allet, B., and Bonnefoy, J. Y. (1995) *J. Biol. Chem.* **270**, 7025–7028
42. Holler, N., Tardivel, A., Kovacsovics-Bankowski, M., Hertig, S., Gaide, O., Martinon, F., Tinel, A., Deperthes, D., Calderara, S., Schultess, T., Engel, J., Schneider, P., and Tschopp, J. (2003) *Mol. Cell. Biol.* **23**, 1428–1440
43. Carbonnelle, D., Ebstein, F., Rabu, C., Petit, J. Y., Gregoire, M., and Lang, F. (2005) *Eur. J. Immunol.* **35**, 546–556
44. Salih, H. R., Schmetzer, H. M., Burke, C., Starling, G. C., Dunn, R., Pelka-Flesicher, R., Nuessler, V., and Kiener, P. A. (2001) *J. Immunol.* **167**, 4059–4066