Attenuating Lymphocyte Activity

THE CRYSTAL STRUCTURE OF THE BTLA-HVEM COMPLEX

Received for publication, July 13, 2005, and in revised form, September 1, 2005 Published, JBC Papers in Press, September 16, 2005, DOI 10.1074/jbc.M507629200

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Five CD28-like proteins exert positive or negative effects on immune cells. Only four of these five receptors interact with members of the B7 family. The exception is BTLA (B and T lymphocyte attenuator), which instead interacts with the tumor necrosis factor receptor superfamily member HVEM (herpes virus entry mediator). To better understand this interaction, we determined the 2.8-Å crystal structure of the BTLA-HVEM complex. This structure shows that BTLA binds the N-terminal cysteine-rich domain of HVEM and employs a unique binding surface compared with other CD28-like receptors. Moreover, the structure shows that BTLA recognizes the same surface on HVEM as gD (herpes virus glycoprotein D) and utilizes a similar binding motif. Light scattering analysis demonstrates that the extracellular domain of BTLA is monomeric and that BTLA and HVEM form a 1:1 complex. Alanine-scanning mutagenesis of HVEM was used to further define critical binding residues. Finally, BTLA adopts an immunoglobulin 1-set fold. Despite structural similarities to other CD28-like members, BTLA represents a unique co-receptor.

Co-receptor signaling is an important mechanism of coordinating and tightly regulating immune response. For instance, activation of naïve T cells requires a second co-stimulatory signal in addition to stimulation of the T cell receptor by engagement with peptide-MHC complexes. Conversely, co-inhibitory signals are required to maintain T cell self-tolerance and prevent autoimmunity (1). The CD28-like family is one important class of co-receptors. These members of the immunoglobulin superfamily (IgSF)2 function as either co-stimulators (CD28 and inducible T cell costimulator) or co-inhibitors (CTLA-4, programmed death-1, and BTLA) in modulating immune cell activity (2). In general, these co-receptors are activated by members of the Ig containing B7 family (1). In addition to the CD28- and B7-like families of receptors and ligands, members of the TNF superfamilies of ligands and receptors (the TNFSF and TNFRSF respectively), such as OX40L-OX40, LIGHT-HVEM, CD27L-CD27, CD30L-CD30, and 4-1BBL-4_1BB, have also been reported to function as co-stimulators (3).

Recently the CD28 family member BTLA was unexpectedly shown to bind and be activated by the TNFRSF member herpes virus entry mediator (HVEM, also known as TNFRSF14, HveA, ATAR, TR2, or LIGHTR) (4, 5). This is the first example of cross-talk between the CD28 family and the TNFRSF. Whereas HVEM has been previously described as a co-stimulator triggered by the TNF-like ligands lymphotoxin α (LTα) and LIGHT (6), recent results from HVEM knock-out mice as well as the interaction between BTLA and HVEM are consistent with HVEM playing a co-inhibitory role (7). In addition to binding BTLA, LIGHT, and LTα, human HVEM is also a host cell receptor for herpes simplex virus 1 by binding to herpes simplex virus 1 glycoprotein D (gD) (8).

Structurally, the connection between the IgSF family represented by BTLA and the TNFRSF proteins such as HVEM is unexpected. Crystal structures of CD28, CTLA-4, and programmed death-1 have revealed that these co-stimulatory and co-inhibitor receptors are all members of the immunoglobulin superfamily with each protein containing an extracellular IgV domain (9–11). Based on sequence analysis, BTLA was also expected to contain an extracellular IgV domain. Similarly, the extracellular domains of B7-like proteins are comprised of Ig domains. Co-crystal structures of B7-1 and B7-2 bound to CTLA4 show that Ig domains from the receptor and ligand pack against each other forming a compact interface (12, 13). In contrast, the TNFSF and TNFRSF members are formed by very different structural elements and interact in a distinctive manner determined by the quaternary structure of TNF-like ligands. These proteins are homotrimERIC or occasionally heterotrimERIC proteins comprised of jelly-roll monomers. Multidomain TNFRSF family members are comprised of multiple pseudo-repeats of a cysteine-rich motif. Structures of signaling complexes formed by TNF-like ligands and receptors show that the elongated receptors bind at monomer-monomer interfaces on the ligands in a manner much different from the compact B7-CD28-type interaction (14).

The BTLA-HVEM interaction is also unusual in that it represents the first example of a TNFRSF functioning as a “ligand” and is one of a handful of examples of a TNFRSF interacting with a non-TNF-like ligand. In addition to the BTLA-HVEM and gD-HVEM interactions, other TNFSF, which bind unusual ligands, include the low affinity neurotrophin receptor p75, which binds the cystine knot growth factor NGF, and feline OX40, which acts as a co-receptor for the feline immunodeficiency virus (15, 16). Crystal structures of the relevant complexes show that gD protein interacts primarily with the N-terminal cysteine-rich domain (CRD1) of HVEM on the surface opposite the TNFSF binding site (17). Similarly, p75 uses the same respective surface on CRD1 and a part of CRD2 to bind NGF (18). Previous biochemical characterization suggests that BTLA also binds to HVEM on the CRD1 distal to the TNFSF binding site (4, 5).

To obtain a more detailed understanding of the novel interaction between the CD28-like protein BTLA and the TNFRSF member HVEM, we have determined the 2.8-Å crystal structure of the BTLA-HVEM complex. This structure shows that despite major structural
Crystal Structure of the BTLA-HVEM Complex

differences between BTLA and gD, they bind to an overlapping site on HVEM using a similar β-sheet binding motif. We have used alanine-scanning mutagenesis and Scatchard assays to identify critical BTLA-binding residues on HVEM. Mutations that significantly reduced binding affinities were in close agreement with the crystal structure. Light scattering demonstrates that the recombinant extracellular domain of BTLA is monomeric and that BTLA and HVEM form a 1:1 complex in solution. Despite the homology and functional similarities between BTLA and the CD28 family, BTLA contains structurally unique features. Moreover, compared with the CD28-B7 binding site, BTLA uses a distinct surface to interact with HVEM. Finally, using the BTLA-HVEM structure, we propose a hypothetical model for a BTLA-HVEM-TNF ternary complex.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification—DNA encoding human BTLA residues 26–137 (the initial methionine is residue 1) with the addition of a C-terminal His$_6$ tag was expressed in *Escherichia coli*. Inclusion bodies from BTLA expressing *E. coli* were extracted under denaturing conditions, and the protein was purified on a Ni-NTA metal chelate column as described (19). Fractions were pooled and diluted to 50 μg/ml with buffer containing 0.1 M Tris (pH 8.6), 0.3 M NaCl, 20 mM glycine, 1 mM EDTA, 1 mM glutathione (oxidized), and 1 mM glutathione (reduced). The refolding mixture was incubated overnight at 2–8 °C, and the pH adjusted to pH 3.0 with trifluoroacetic acid. The acidified refolding mixture was loaded onto an RP-HPLC Vydac C4 column (1.0 × 25 cm) equilibrated with 0.1% (w/v) trifluoroacetic acid in water and eluted with a linear gradient of acetonitrile (from 15 to 55%) in 0.1% trifluoroacetic acid at 3 ml/min for a total of 35 min. Protein fractions were pooled, and the acetonitrile was removed by evaporation assisted by a gentle stream of N2. The RP-HPLC pool was buffer exchanged using a HiTrap Desalting column (Amersham Biosciences) equilibrated with buffer containing 10 mM HEPES (pH 6.8), 0.15 M NaCl. BTLA activity was evaluated using SPR (Biacore).

BTLA-Fc fusion protein was expressed in Chinese hamster ovary cells as previously described (4). The BTLA-Fc construct contains a Genenase site (New England Biolabs) between the BTLA extracellular domain (ECD) and the Fc fusion. BTLA ECD was cleaved from the Fc domain by the addition of a 1:100 ratio of Genenase to BTLA-Fc in glycine, 1 mM EDTA, 1 mM glutathione (oxidized), and 1 mM glutathione (reduced). The refolding mixture was incubated overnight at 2–8 °C, and the pH adjusted to pH 3.0 with trifluoroacetic acid. The acidified refolding mixture was loaded onto an RP-HPLC Vydac C4 column (1.0 × 25 cm) equilibrated with 0.1% (w/v) trifluoroacetic acid in water and eluted with a linear gradient of acetonitrile (from 15 to 55%) in 0.1% trifluoroacetic acid at 3 ml/min for a total of 35 min. Protein fractions were pooled, and the acetonitrile was removed by evaporation assisted by a gentle stream of N2. The RP-HPLC pool was buffer exchanged using a HiTrap Desalting column (Amersham Biosciences) equilibrated with buffer containing 10 mM HEPES (pH 6.8), 0.15 M NaCl. BTLA activity was evaluated using SPR (Biacore).

BTLA-HVEM complex was made by adding excess HVEM residues 1–105 with an S-75 sizing column equilibrated in 150 mM NaCl, 20 mM Tris (pH 8.0). Fractions containing purified complex were pooled and concentrated to 9 mg/ml.

DNA encoding LIGHT extracellular domain residues 91–240 was expressed and purified in the same manner as HVEM with a final purification on Superdex S-200 sizing equilibrated in 150 mM NaCl, 20 mM Tris (pH 8.0). Expression yielded greater than 1 mg of purified protein/liter of Hi5 cells.

Crystallographic Data Collection and Structure Determination—Crystals of the BTLA-HVEM complex were grown by vapor diffusion at 19 °C using the sitting drop method. Crystals formed in drops containing protein solution were mixed with an equal volume of reservoir solution containing 2.0 M sodium formate, 0.1 M sodium acetate (pH 4.6). The resulting small, clustered crystals were used to seed new drops yielding larger, single crystals. The crystals were transferred briefly to a droplet containing reservoir solution with 30% glycerol before flash-freezing in liquid nitrogen. The crystals belonged to space group C2221, and the asymmetric unit contained two copies of the BTLA-HVEM complex. A data set to 2.8-Å resolution was measured from a single crystal at beam line 5.0.1 of the Berkeley Center for Structural Biology at the Advanced Light Source. The data were processed using the HKL package (22).

Structures of HVEM (chain B in PDB code 1JMA) and murine BTLA (PDB 1XAU; structure determined and deposited by D. Fremont and co-workers in 2004) were used as search models to determine the structure of the BTLA-HVEM complex by molecular replacement. Side chains, which differed between murine and human BTLA, were manually trimmed to C-β. The program Phaser (23) gave a clear solution. A 2-fold noncrystallographic symmetry-averaged and solvent-flattened map using program dm (24) revealed clear density for the missing hBTLA side chains. The model was refined with REFMAC5 (25) with tight noncrystallographic symmetry restraints on residues 34–144 of BVEM and residues 4–83 of HVEM. The last 10 residues of BTLA were not included in the noncrystallographic symmetry restraints as they differed significantly because of the involvement of the C-terminal His tag in crystal packing contacts. Additional density was observed for a metal ion interacting with the His tag of symmetry related copies of chain A. This ion was modeled as a Ni(II) and is likely an artifact because of purification of BTLA by Ni-NTA affinity chromatography. Refinement and model statistics are shown in Table 1. The coordinates for the BTLA-HVEM complex have been deposited in the Protein Data Bank and assigned access code 2AW2.

Alanine Scanning Mutagenesis and Cell Binding Affinity Assays—The QuikChange site-directed mutagenesis kit (Stratagene) was used as recommended by the manufacturer to generate single alanine mutations in HVEM. Mutant HVEM sequences were confirmed by DNA sequencing. Recombinant BTLA-Fc was iodinated by the lactoperoxidase (Biotrend) method and LIGHT by the IODO-GEN (PerkinElmer Life Sciences) method. Displacement binding studies were done as previously described (4) with 0.5 nM labeled BTLA and varying concentrations of unlabeled protein. LIGHT binding was confirmed with 0.5 nM labeled LIGHT with or without 1000-fold excess of unlabeled protein. LIGHT binding to alanine mutants was normalized as a percentage of wild type binding. AD-293 cells were transiently transfected with either
wild type or alanine mutant HVEM cDNA as previously described (4) using pRK mock-transfected cells as a negative control. Expression of wild type and alanine mutant HVEM was confirmed by flow cytometry as previously described using fluorescein isothiocyanate-conjugated mouse anti-human HVEM (clone 122, MBL) in comparison to an isotype control (BD Biosciences). The HVEM antibody epitope is contained in all HVEM point mutants. Expression levels of point mutants are normalized to that of wild type HVEM as denoted by the percentage of cells above a given mean fluorescence threshold determined by isotype control antibody staining.

Light Scattering—Molar Mass determination was carried out using an Agilent 1100 series (Agilent, Palo Alto, CA) HPLC system in line with a Wyatt MiniDawn MALS (multisite light scattering) detector (Wyatt Technology, Santa Barbara, CA). Concentration measurements were made using an online Wyatt OPTILAB DSP interferometric refractometer (Wyatt Technology). Astra software (Wyatt Technology) was used for light scattering data acquisition and processing. Either a Shodex 803 or a S75 10/300 column (Amersham Biosciences) equilibrated with filtered phosphate-buffered saline (pH 7.2) was used with a flow rate of 1 ml/min. Both the light scattering unit and the refractometer were calibrated as per the manufacturer’s instructions. A value of 0.180 ml/g was assumed for the dn/dc ratio of the protein. Measuring the signal from monomeric bovine serum albumin normalized the detector responses.

The temperature of the light scattering unit was maintained at 25 °C, and the temperature of the refractometer was kept at 35 °C. The column and all external connections were at ambient temperature (20–25 °C). Recombinant BTLA produced in Chinese hamster ovary cells, the purified HVEM-BTLA complex used for crystallization, and HVEMΔC were loaded at 1.0 mg/ml.

RESULTS

The structure of the human BTLA-HVEM complex was solved by molecular replacement using the structures of the HVEM ECD (PDB code 1JMA, chain B) and the 1.8-Å structure of murine BTLA ECD (PDB code 1XAU) as search models with the program Phaser (23). A solution was found with two copies of the BTLA-HVEM complex forming the asymmetric unit. The structure was manually rebuilt and refined to an R/Rfree of 23.1 and 27.8%, respectively (TABLE ONE, Fig. 1). The final model consists of BTLA residues 34–137 and HVEM residues 5–92 and 95–102. In both copies of BTLA, an additional 4–6 residues from the C-terminal His tag were well ordered in the electron density and are included in the model. Noncrystallographic symmetry restraints were used throughout the refinement and the two copies of the BTLA-HVEM complex are very similar.

This structure reveals that human BTLA, like murine BTLA (PDB code 1XAU), is a compact IgG domain composed of two flat β-sheets, which are formed by strands B, E, and D in one sheet and strands A’, G, F, C, and C’ in the other (Fig. 1B). The sheets are buttressed by three disulfides (residues 72–79, 34–63, and 58–115). The Cys57-Cys79 disulfide connects the C and C’ strands; the Cys34-Cys63 disulfide joins the N-terminal region preceding strand A’ to the CD loop, and the Cys58-Cys115 disulfide connects the B and F strands. This disulfide is completely buried in the hydrophobic core of BTLA and is part of the “Y-corner” motif. DXG(A/D)DXYXC. The B–F strand disulfide and the Y-corner motif are both highly conserved features in IgSF domains. In addition to the Cys58-Cys115 disulfide, the Cys34-Cys63 disulfide is also conserved in murine BTLA. The Cys72-Cys79 disulfide is not conserved in all murine BTLA alleles (see below). This disulfide is not present in the variant of murine BTLA, which was crystallized (PDB code 1XAU) in which the cysteine corresponding to Cys79 is replaced by a tryptophan.

The sequence of human BTLA contains three putative N-linked glycosylation sites. Expression of recombinant BTLA in eukaryotic cells results in a protein with significant glycosylation that is unsuitable for crystallization. For the structural studies, recombinant BTLA was expressed in E. coli cells to produce a protein without glycosylation. Examination of the BTLA-HVEM complex (below) shows that the putative N-linked glycosylation sites are away from the binding site and would not be expected to affect the interaction between BTLA and HVEM (Fig. 1). Analysis of the interaction between BTLA expressed in E. coli and HVEM by surface plasmon resonance shows that glycosylation is not required for HVEM binding.

The BTLA-HVEM Complex—The BTLA-HVEM complex consists of a single globular BTLA interacting with the membrane distal region of rod-shaped HVEM (Fig. 1). BTLA binds HVEM using two short segments: an N-terminal extension preceding strand A’ (residues 35–43) and the short G’ strand (residues 118–128). The HVEM binding site for BTLA consists almost exclusively of residues from CRD1. BTLA residues 35–43 interact with HVEM residues 26–33, which form the “tip” of HVEM CRD1 distal to the C terminus. This loop in HVEM leads to a strand (residues 33–38) that, together with the G’ strand from BTLA, makes the heart of the binding interface. These two strands form a small anti-parallel intermolecular β-sheet. This interaction is primarily mediated by main chain hydrogen bonds and includes relatively few side chain contacts. These two interactions, in conjunction with small contribution from the BTLA CC’ loop, generate an interface that bury ~1800 Å² of solvent accessible surface area, which is contributed equally by both binding partners. This complex positions the C termini of the two proteins in opposite directions consistent with the BTLA-HVEM complex forming between proteins resident on different cells.

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**TABLE ONE**

| Crystallographic data collection and refinement statistics |
|----------------------------------------------------------|
| **BTLA-HVEM**                                           |
| Data collection                                         |
| Space group C222, Resolution (Å) 50–2.80 (2.90–2.80)* |
| Unit cell constants (Å)                                 |
| a = 50.5 b = 168.2 c = 149.4                           |
| Rsym (%)                                               |
| 0.052 (0.455)*                                         |
| No. observations                                       |
| 62,260                                                 |
| Unique reflections                                     |
| 15,639                                                 |
| Completeness (%)                                       |
| 99.9 (100)*                                            |
| (I/σI)                                                 |
| 13.4 (2.3)*                                            |
| Asymmetric unit 2 BTLA-HVEM complexes                   |
| Refinement                                             |
| Resolution (Å)                                         |
| 30–2.8                                                 |
| Final R/Rfree (%)                                      |
| 23.1, 27.8                                             |
| Root mean square deviation bonds (Å)                    |
| 0.007                                                  |
| Root mean square deviation angles (°)                   |
| 1.04                                                   |
| Root mean square deviation bonded B (Å²)                |
| 1.8                                                    |
| Ramachandran plot (%)                                   |
| 86.5; 12.4; 0.8; 0.3                                    |

* Numbers in parentheses refer to the highest resolution shell.

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L. Gonzalez, unpublished data.
white (CRD1, bonding pattern formed at the interface. Residues of interest are labeled. C-

Moreover, as full-length HVEM includes an additional ~60 residues prior to the transmembrane helix, it is also possible that the binary BTLA-HVEM interaction could occur between proteins residing on the same cell.

**Sequence Polymorphism in Murine BTLA**—Three different BTLA alleles have been isolated from 23 mice strains. These alleles have been labeled BALB/c-like, MRL/lpr-like, and C57BL/6-like according to the strains from which they were derived (26). The crystal structure of murine BTLA is of the BALB/c-like variant. The BALB/c and MLR/lpr-like, and C57BL/6-like alleles have been isolated from 23 mice strains. These alleles have been characterized based on the severity of the effect of the alanine substitution. Substitution at positions Pro17, Tyr23, and Val36 had a more pronounced effect than substitutions at positions Glu8, Lys26, and Val31. Variant proteins with alanine at either Pro17 or Tyr23 had no detectable affinity for BTLA, although LIGHT binding was largely intact indicating that the HVEM fold was not seriously compromised. The side chains of these two residues pack against either end of the intermolecular bridge with a potential hydrogen bonding interaction. Three of the remaining differences, E41P(h35), N45T(h39), and K47T(h41), affect residues in the vicinity of the expected receptor binding site but are not predicted to make significant contacts to murine HVEM. The remaining polymorphisms in murine BTLA occur outside the HVEM binding site and are not expected to affect either BTLA structure or HVEM affinity. In summary, because none of the side chains of any of the variant residues are predicted to be in intimate contact with murine HVEM, all three alleles are expected to code for proteins with comparable affinity for HVEM.

**Functional Characterization of the BTLA-HVEM Interface**—Alanine scanning mutagenesis (27) was used to identify the functional epitope on HVEM for BTLA. Based on prior characterization of this complex (4), 15 residues were selected for mutagenesis based on three criteria: conservation in murine and human BTLA, location on the side of HVEM away from the expected TNFSF binding site, and solvent exposure (TABLE TWO). Single alanine-substituted variants of HVEM were expressed transiently in AD-293 cells, and BTLA binding data were measured for Scatchard analysis. Six residues (Glu8, Pro17, Tyr23, Lys26, Glu31, and Val36) showed greater than 2-fold reduction in affinity when mutated to alanine (Fig. 2). These six residues can be divided into two groups based on the severity of the effect of the alanine substitution. Substitution at positions Pro17, Tyr23, and Val36 had a more pronounced effect than substitutions at positions Glu8, Lys26, and Val31. Variant proteins with alanine at either Pro17 or Tyr23 had no detectable affinity for BTLA, although LIGHT binding was largely intact indicating that the HVEM fold was not seriously compromised. The side chains of these two residues pack against either end of the intermolecular β-sheet likely providing crucial van der Waals contacts. Mutation of HVEM residue Val36 to alanine resulted in a 10-fold reduction in BTLA affinity. The Val36 side chain packs against BTLA residue Ile124, whereas the backbones of these two residues form reciprocal anti-parallel hydrogen bonding interactions.

**TABLE TWO**

| HVEM | BTLA $K_d$ (nM) | Error | LIGHT binding | Expression level |
|------|----------------|-------|---------------|-----------------|
| WT   | 25             | 3     | 1.00          | 1.00            |
| E8A  | 89             | 18    | 1.23          | 0.95            |
| E14A | 40             | 3     | 1.53          | 0.87            |
| P17A | ND             | 0.09  | 0.89          | 0.89            |
| Y23A | ND             | 0.69  | 0.77          | 0.77            |
| K26A | 71             | 7     | 0.97          | 1.00            |
| E31A | 95             | 17    | 1.59          | 0.83            |
| T33A | 33             | 5     | 0.97          | 0.86            |
| V36A | 241            | 79    | 0.88          | 1.02            |
| P39A | 43             | 5     | 1.21          | 0.80            |
| S74A | 34             | 4     | 0.78          | 0.97            |
| V80A | 24             | 2     | 0.30          | 0.86            |
| P85A | 21             | 12    | 0.41          | 0.52            |
| F88A | 25             | 1     | 0.86          | 1.07            |
| Q92A | 32             | 2     | 1.32          | 0.97            |
| H96A | 27             | 2     | 1.21          | 0.84            |
| Vector | ND          | 0.06  | 0.08          |                 |

Human HVEM and point mutans were expressed in AD-293 cells and binding studies were carried out with radiolabeled recombinant BTLA-Fc or LIGHT. $K_d$ values were determined by Scatchard analysis. LIGHT binding and HVEM expression levels are normalized to the wild type level. Expression level was determined by flow cytometry. ND, not detectable.
FIGURE 2. Alanine-scanning mutagenesis of HVEM. a, mutated residues that reduced BTLA binding >5-fold are colored red; >2-fold, yellow; and unchanged binding, green. b, the BTLA-binding site on HVEM is colored blue. BTLA is shown as a C-α rendering in yellow. c and d, representative displacement curves and Scatchard plot for BTLA-Fc binding wild type HVEM. e and f, representative displacement curve and Scatchard plot for BTLA-Fc binding the K26A HVEM mutant.
bonds at the center of the intermolecular anti-parallel \( \beta \)-sheet. Significantly, the three most disruptive alanine substitutions were located at residues involved in the formation of the HVEM-BTLA intermolecular \( \beta \)-sheet.

Alanine substitutions at Glu\(^8\), Lys\(^{26}\), and Glu\(^{31}\) all caused a \( \sim \)3-fold decrease in HVEM affinity for BTLA. These residues are located at the periphery of the binding interface (Fig. 2). Alanine mutation at nine other residues had no effect on BTLA binding. Of these residues, only Thr\(^{33}\) and Pro\(^{39}\) bury more than 50% of their accessible surface area in the BTLA-HVEM interface. Thus the alanine scanning and structural data both indicate that the anti-parallel strand and immediately surrounding interactions are the energetic and structural core of the BTLA-HVEM interface.

**FIGURE 3. BTLA-HVEM is a heterodimer in solution.**

- **a.** the dimer of dimers forming the crystallographic asymmetric unit of BTLA-HVEM. BTLA and HVEM are colored as in Fig. 1a.
- **b.** representative light scattering data showing glycosylated BTLA ECD is monomeric in solution with apparent molecular mass of 22,000 g/mol. C, representative light scattering data showing that the ECD of BTLA and HVEM form a heterodimeric complex in solution at a concentration of 40 \( \mu \)m apparent molar mass of 24,600 g/mol. HVEM\(_{ECD}\) (the entire HVEM ECD with a C-terminal His tag) and the soluble complex between HVEM\(_{ECD}\) and glycosylated BTLA were also assessed using this procedure. Soluble HVEM\(_{ECD}\) was found to be monomeric with an apparent molecular mass of 12,400 g/mol, whereas the HVEM\(_{ECD}\)-glycosylated BTLA complex appears heterodimeric with an approximate molecular mass of 32,400 g/mol. The apparent molecular mass of the HVEM\(_{ECD}\) and the HVEM\(_{ECD}\)-glycosylated BTLA complexes are slightly lower than predicted likely because of proteolytic degradation of the C-terminal portion of HVEM\(_{ECD}\).

**BTLA-HVEM Complex Forms a Stable Heterodimer in Solution**—The crystallographic asymmetric unit contains two BTLA-HVEM complexes forming a dimer of dimers (Fig. 3). To ascertain the relevance of this interaction, the stoichiometry of the BTLA-HVEM complex in solution was characterized by multiangle light scattering. This analysis showed that the complex formed by *E. coli*-derived BTLA and truncated HVEM ECD, which was used for crystallography, as well as the complex of Chinese hamster ovary-derived glycosylated BTLA with the entire HVEM ECD both form stable heterodimers with no indication of higher order assemblies in solution at a concentration of \( \sim \)40 \( \mu \)M (Fig. 3). In addition, both glycosylated and *E. coli*-derived BTLA were found to be monomeric in solution. This data, in combination with the relatively small area of contact between the adjacent heterodimers in the crystal-
Whereas gD and BTLA both bind HVEM and compete for the same site on HVEM, their structures are strikingly different (Fig. 4). gD is formed by a core Ig domain that is decorated with a N-terminal extension (~50 residues) lacking regular secondary structure and a longer partially helical C-terminal extension (~100 residues). The gD Ig domain does not contact HVEM directly. Instead, the N-terminal extension forms most of the contacts to HVEM and is supported by an α-helix from the C-terminal extension (17). In contrast, BTLA consists solely of an Ig domain and uses this domain to bind HVEM. Despite these differences, BTLA and gD have converged on a very similar structural and energetic solution for specifically binding HVEM.

This convergence suggests that formation of this anti-parallel intermolecular β-sheet is a favorable interaction. Intriguingly, the HVEM strand (28, 29) at the heart of the BTLA and gD bonding sites is one of the relatively few conserved elements of secondary structure in the TNFRSF-fold. This strand is present in both death receptor-5 (30, 31) and TNFR1 (32), the only other multidomain TNFRSF family members that have been structurally characterized. In TNFR1, the strand is present but shorter than in HVEM. The structure of death receptor-5, which has a truncated CRD1, retains a diminished strand at this position although it is occluded by the death receptor-5 N terminus. At the sequence level, Thr35–Val36 is present at this position in ~30% of multidomain TNFRSF members and Cys37 is invariant. Position 38 is solvent exposed and is not conserved, whereas Pro39 is present in ~60% of multidomain TNFRSF sequences. This conservation of sequence and structure suggests that other TNFRSF could interact with non-TNFRSF binding partners by forming a similar anti-parallel β-sheet binding motif.

**BTLA Is a Member of the I-set of IgSF Domains**—In contrast to previous suggestions based on sequence analysis (33), examination of the BTLA ECD structure indicates that it belongs to a different subset of the IgSF than the CD28-like family. IgSF domains can be divided into 4 main classes, the Variable (V), Constant 1 (C1), Constant 2 (C2), and Intermediate (I) sets, based on their structures and sequences (34). One of the distinguishing features of I-set domains is the lack of a C′ strand. The present structure shows that the BTLA ECD domain lacks this strand and thus is better described as a member of the I-set of Ig domains. Examination of 20 “fingerprint” residues, which differentiate I-set and V-set Ig domains from other Ig domains (34), confirms this analysis (Fig. 5). The structures of CTLA4 (10), programmed death-1 (11), and CD28 (9) as well as the sequence of inducible T cell costimulator indicate that these proteins all possess a C′ strand and are better described as members of the V-set of Ig domains. Furthermore, these four known CD28-like proteins are all located in close proximity on chromosome 2, whereas BTLA is located separately on chromosome 3. This analysis, as well as the fact that BTLA binds a structurally different ligand, strongly suggests that BTLA is distinct from the CD28 family.

**BTLA Utilizes a Distinct Binding Surface Compared with CD28 Family Members**—The structures of CTLA4 bound to B7-1 and B7-2, which is expected to be representative of CD28 family interactions, shows that the conserved MYPPPYYL sequence in the CTLA4 GF-loop, in conjunction with residues on the C and F strand, form a relatively flat
binding surface (12, 13). In contrast, the BTLA binding surface is located along the edge of the I-set Ig domain almost orthogonal to the CTLA4 binding surface (Fig. 5). Even more strikingly, the CTLA4-B7 complexes suggests that at least some of these proteins are capable of forming a periodic repeating array at the immunological synapse. For instance, a covalent “outward” facing CTLA4 dimer can interact with two separate B7-like dimers (Fig. 6). This type of interaction may lead to the clustering of a large number of CTLA4 molecules resulting in high local intracellular concentrations of ITIM or ITAM domains (12, 13). In contrast, the BTLA binding surface is located in both structure and binding mode, than had been expected from the alignment. The Cα and Cβ strands in CTLA4 and CD28 are labeled with green italics. A blue asterisk appears above every fifth BTLA residue. The 20 V-frame fingerprint residues are indicated with black dots. Residues that fulfill this criterion are in bold. Orange shading, BTLA residues that bury at least 25% of accessible surface area upon binding HVEM. Green shading, residues in CTLA4 that bury at least 25% of accessible surface area upon binding B7-1. Predicted glycosylation sites in hBTLA are boxed in purple. Murine BTLA residues that differ in BALB/c-like, MLP/CD28 and CTLA4, which is missing in BTLA, is boxed. b, molecular surface of BTLA in the same orientation as in a. Residues forming the BTLA-HVEM interface are colored by % accessible surface area buried upon complex formation (25–50%, yellow; 50–75%, orange; 75–100%, red). c, molecular surface of CTLA4 in the same orientation as in a. Residues forming the CTLA4-B7-1 interface are colored as described for BTLA. d, sequence alignment of Ig domains of human BTLA, murine BTLA, human CD28, and human CTLA4. Secondary structure of hBTLA is shown above the alignment. The Cα and Cβ strands in CTLA4 and CD28 are labeled with green italics. A blue asterisk appears above every fifth BTLA residue. The 20 V-frame fingerprint residues are indicated with black dots. Residues that fulfill this criterion are in bold. Orange shading, BTLA residues that bury at least 25% of accessible surface area upon binding HVEM. Green shading, residues in CTLA4 that bury at least 25% of accessible surface area upon binding B7-1. Predicted glycosylation sites in hBTLA are boxed in purple. Murine BTLA residues that differ in BALB/c-like, MLP/CD28, and C57BL/6-like alleles (26) are underlined.

**Conclusions**—The BTLA-HVEM complex simultaneously provides examples of both convergent and divergent evolution and demonstrates the plasticity of evolutionary relationships determining protein-protein interactions. Unexpectedly, our data showed that two structurally very different proteins, BTLA and gD, have converged not just on the same binding site but also use similar elements of secondary structure to bind HVEM. In addition, the BTLA-HVEM structure further differentiates BTLA from the CD28 family of proteins. BTLA and CTLA4 differ more, in both structure and binding mode, than had been expected from amino acid sequence. BTLA may have diverged from the CD28-like complex has not been determined. As LIGHT is not required for BTLA activation, the physiological relevance of a ternary complex is unclear at this time (7, 33). A model of a possible BTLA-HVEM-LTα ternary complex was generated by assuming that the LTα-HVEM complex will be very similar in geometry to the LTα-TNFR1 complex (32). The geometry of this complex model predicts that LIGHT and BTLA would be expressed on the same cell, whereas HVEM would be expressed on a different cell (Fig. 6b). Finally, because BTLA does not promote trimeric clustering of HVEM, it is unlikely to have the same effect on HVEM activation as LIGHT binding, which causes three copies of HVEM to cluster and trigger cytosolic signal transduction.

**Implications for BTLA-HVEM-Ligand Ternary Complexes and HVEM Signaling**—In addition to binding BTLA, HVEM is also known to bind TNFSF family members LIGHT and LTα. BTLA binding does not occlude the TNFSF binding site on the opposite face of HVEM and thus HVEM should be able to simultaneously bind BTLA and either LIGHT or LTα to form a stable ternary complex. We have been able to purify a complex containing recombinant LIGHT, HVEM, and BTLA by size exclusion chromatography although the stoichiometry of this complex has not been determined. As LIGHT is not required for BTLA activation, the physiological relevance of a ternary complex is unclear at this time (7, 33). A model of a possible BTLA-HVEM-LTα ternary complex was generated by assuming that the LTα-HVEM complex will be very similar in geometry to the LTα-TNFR1 complex (32). The geometry of this complex model predicts that LIGHT and BTLA would be expressed on the same cell, whereas HVEM would be expressed on a different cell (Fig. 6b). Finally, because BTLA does not promote trimeric clustering of HVEM, it is unlikely to have the same effect on HVEM activation as LIGHT binding, which causes three copies of HVEM to cluster and trigger cytosolic signal transduction.
Crystal Structure of the BTLA-HVEM Complex

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Acknowledgments—We thank F. Arellano for providing glycosylated BTLA, R. Corpuz for purifying BTLA, H. Raab and M. Elliot for the laser-light scattering data, R. Vandau for support, M. Franklin and the staff at ALS beamline S.0.1 for assistance with data collection, C. Wiesmann for advice on IgSF domains, Charles Eigenbrot for comments on the manuscript, and our colleagues in mass spectrometry and protein sequencing.

Addendum—While this manuscript was under review, Cheung et al. (35) published a more limited mutagenesis study on the HVEM-BTLA interaction. They report that the K26A mutation in HVEM affects BTLA binding and conclude that the BTLA binding site is centered around HVEM residues 24–27 and is overlapping but distinct from the gD binding site. This interpretation is inconsistent with the crystal structure and more elaborate mutagenesis results reported here.

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FIGURE 6. The BTLA-HVEM signaling complex. a, contrast between the heterodimeric BTLA-HVEM complex and repetitive zipper model proposed for CTLA4-B7 interactions (12, 13). b, a model of the ternary complex formed by BTLA-HVEM (yellow and blue) and either LIGHT or LTα (gray) was constructed by superimposing the HVEM portion of the BTLA-HVEM complex on the TNFR1 molecule in the LTα-TNFR1 complex (32) and using 3-fold symmetry to generate the full complex. The portion of HVEM, which is disordered, is shown as an oval.