



The three HveA receptor ligands, gD, LT- α and LIGHT bind to distinct sites on HveA

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Abstract

The herpes virus entry mediator A (HveA), a member of the tumor necrosis factor receptor (TNFR) superfamily, interacts with three different protein ligands; lymphotoxin- α (LT- α) and LIGHT (LIGHT stands for lymphotoxin homolog, which exhibits inducible expression and competes with HSV glycoprotein D for HveA and is expressed on T-lymphocytes) from the host and the herpes simplex virus (HSV) surface glycoprotein gD. It has been reported that the gD binding site on HveA is located within the receptor's two N-terminal CRP domains, and that gD and LIGHT compete for their binding to HveA. However, whether these ligands interact with the same or different sites on the receptor is unclear. We analyzed and compared the sites of interaction between HveA and its TNF ligands, by using two recombinant forms of the receptor, comprising the full-receptor ectodomain (HveA (200t)) and its two first CRP domains (HveA (120t)), as well as several monoclonal antibodies recognizing HveA. Two HveA peptide ligands (BP-1 and BP-2) that differentially inhibit binding of soluble gD and LT- α to the receptor were also used to demonstrate that gD, LIGHT and LT- α bind to distinct sites on the receptor. Our results suggest that binding of a ligand to HveA may alter the conformation of this receptor, thereby affecting its interaction with its other ligands. © 2001 Published by Elsevier Science Ltd.

Keywords: Herpes simplex virus; gD; Herpes virus entry mediator A; LIGHT; Lymphotoxin- α ; Tumor necrosis factor receptor

1. Introduction

The proteins of the tumor necrosis factor receptor (TNFR) family participate in several key biological processes, including the development and proper function of host innate and adaptive immunity (Smith et al., 1994; Kwon et al., 1999). One of these proteins, the herpes virus entry mediator A (HveA, formerly HVEM), is a 283-amino acid type I integral membrane

protein, which contains a motif of four cysteine-rich pseudorepeat TNFR domains (Montgomery et al., 1996), and appears to play a role in the immune response by modulating T-cell proliferation. Monoclonal antibodies recognizing HveA block T-cell proliferation, as well as the expression of activation markers and release of cytokines by these cells (Harrop et al., 1998). This receptor can be found in many tissues, but is most abundant in the spleen and peripheral blood leukocytes (Mauri et al., 1998; Kwon et al., 1999, 1997; Montgomery et al., 1996).

HveA interacts with two proteins of the TNF family, lymphotoxin- α (LT- α), and LIGHT (LIGHT stands for lymphotoxin homolog, which exhibits inducible expres-

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sion, competes with HSV glycoprotein D for HveA and is expressed on T-lymphocytes) (Mauri et al., 1998). Studies using LT- α -deficient mice have revealed an important role for this molecule in the formation of secondary lymphoid organs during development (Matsumoto, 1999; Kwon et al., 1999; Smith et al., 1994). Because LT- α interacts with the TNFR family proteins TNFR1 and TNFR2 as well as HveA, the specific role of the LT- α -HveA interaction is yet unknown. Likewise, LIGHT has been shown to interact with the lymphotoxin β receptor (LT β R) as well as HveA (Mauri et al., 1998; Rooney et al., 2000; Zhai et al., 1998). LIGHT signaling through the LT β R appears to mediate apoptosis of tumor cells.

HveA also serves as a receptor for herpes simplex virus (HSV), i.e., it is capable of mediating the entry of most HSV-1 and HSV-2 strains into host cells (Montgomery et al., 1996; Whitbeck et al., 1997). This effect is achieved through the direct binding of the viral surface glycoprotein gD to HveA (Whitbeck et al., 1997; Nicola et al., 1998). Furthermore, a soluble form of HSV gD (gDt) specifically competes with membrane anchored LIGHT for binding to HveA, an observation which not only inspired the name of this protein, but also led to the hypothesis that gD may modify HveA-signaling activities during entry or egress of HSV, thus modulating the immune response of the host (Raftery et al., 1999).

We had previously used a recombinant form of HveA to screen phage-displayed combinatorial peptide libraries, from which we selected two peptide ligands (BP-1 and BP-2) that differentially inhibited the binding of gDt and LT- α to the receptor; BP-2 was also able to block HSV entry into HveA-expressing CHO cells (Sarrias et al., 1999). We observed that binding of BP-1 to HveA was inhibited by BP-2, and vice versa, and that LT- α and gD did not compete for binding to the receptor. We proposed that the effects of the BP-1 and BP-2 peptides upon gD and LT- α binding could be mediated either by direct competition for the ligands' binding sites on the receptor, or BP-1 and BP-2 might induce a conformational change in HveA, thereby modifying the domains through which it interacts with gD and LT- α .

Our goal in the present study was to further analyze the interactions between HveA and its cellular ligands, LT- α and LIGHT. To this end, we have characterized the effect of viral gD and peptides BP-1 and BP-2, as well as a truncated form of HveA [HveA (120t)], and monoclonal antibodies recognizing HveA upon the interactions of LT- α and LIGHT with HveA. Our findings indicate that gD, LT- α and LIGHT bind to distinct sites on this receptor and that conformational changes induced by the binding of one ligand to HveA may affect its binding ability for its other ligands.

2. Materials and methods

2.1. Chemicals and buffers

All chemicals and reagents used for peptide synthesis were purchased from Applied Biosystems (Foster City, CA), with the exception of the 9-fluorenylmethoxycarbonyl (F-moc) amino acids, which were obtained from Nova Biochem (San Diego, CA).

2.2. Protein expression

The production and purification of HveA (200t) (Whitbeck et al., 1997), HveA (120t) (Whitbeck et al., 2000), gD-1(Δ 290-299t) (Nicola et al., 1996; Sisk et al., 1994), and LT- α (Crowe et al., 1994), from recombinant baculovirus-infected cells has been described. The protein LIGHT, fused to the FLAG epitope was produced in HEK293 cells and purified from culture supernatants, as described previously (Rooney et al., 2000).

2.3. Antibodies

The production of monoclonal antibodies against HveA (200t) (CW1, CW2, CW3, CW4, CW7 and CW8) has been described (Whitbeck et al., 2000). The antibodies were purified from mouse ascitic fluid using protein G chromatography, and dialyzed in PBS (Whitbeck et al., 2000). A rabbit anti-gD polyclonal antibody (R7) was raised against gD-2 isolated from virus-infected cells, as previously described (Isola et al., 1989). The production of monoclonal antibody (AG9) against LT- α has been described before (Browning et al., 1995).

2.4. Synthesis and purification of peptides

The synthesis and purification of peptides BP-1, BP-1 (4,10 Acm), BP-2, and BP-2 (3,9 Ala) have been previously described (Sarrias et al., 1999). The primary sequences of BP-1 and BP-2 are, SISCSRGLVCLL-PRLTNESGNDRFDS and GSCDGFRCYMH, respectively. All peptides were synthesized on an Applied Biosystems peptide synthesizer (model 431A), using F-moc amide resin. The side chain protecting groups were Cys (Trt), Cys (Acm), Arg (Pmc), Ser (tBu) and Tyr (tBu). The disulfide oxidation of BP-2 was performed on resin, while that of BP-1 was performed after cleavage from the resin. All peptides were purified by reversed-phase high-performance liquid chromatography on an automated system (Prep-LC 4000; Waters, Milford, MA) with a C₁₈ column (Vydac, Western Analytical Products, Murrieta, CA). The purity of the final products was assessed by analytical high-performance liquid chromatography and matrix-assisted laser desorption mass spectrometry, using a time-of-flight

mass spectrometer (Micro-Mass Tofspec; Micromass, Beverly, MA) (Moore, 1997, 1993).

2.5. ELISAs

Several ELISAs were performed to study the interactions between HveA (120t), HveA (200t), gD, LIGHT, LT- α , BP-1 and BP-2. In these assays, microtiter wells were coated for 2 h at 22°C with 40 ng HveA (200t), HveA (120t), or BSA. Non-specific binding to the wells was prevented with the addition of blocking buffer (Phosphate buffered saline (PBS) containing 1% BSA) for 1 h at 22°C. The ELISA assays were performed as follows: (1) For competition assays involving peptides BP-1 and BP-2 or monoclonal antibodies (CW1, CW2, CW3, CW4, CW7 and CW8), serial dilutions were added to the wells and incubated for 30 min at 22°C. Recombinant protein (1.5 nM LIGHT, or 12.5 nM LT- α) or phage supernatant was then added and incubated for 1 h at 22°C. (2) In competition assays, where HveA (200t), HveA (120t) and LT- α were tested in solution, serial dilutions of these proteins were preincubated with either 12.5 nM LT- α or 1.5 nM LIGHT for 30 min at 22°C and then added to the HveA-coated microtiter wells. (3) For the salt-dependence assay, LT- α (25 nM) or LIGHT (3 nM) were diluted in 10 mM phosphate buffer pH 7.4 containing different amounts of NaCl, and subsequently incubated with the HveA-coated wells for 1 h at 22°C. The washing buffer in this assay was 10 mM phosphate buffer, with 50 mM NaCl, and Tween-20 0.05%, pH 7.4.

Next, the plates were washed twice with PBS containing 0.05% Tween-20 and incubated with (i) monoclonal antibody AG9 (1 μ g/ml), (ii) the M2 anti-FLAG monoclonal antibody (2 μ g/ml; Stratagene), or biotinylated M2 anti-FLAG (2 μ g/ml; Sigma), for 1 h at 22°C. Then, the plates were washed twice and incubated for 30 min at 22°C with a 1:1000 dilution of peroxidase-la-

beled (i) anti-M13 antibody (Pharmacia) for phage detection, (ii) anti-mouse immunoglobulin G antibody (Bio-Rad) for monoclonal antibody detection, (iii) anti-rabbit immunoglobulin G antibody (Bio-Rad) for polyclonal antibody detection, or (iv) streptavidin (Boehringer Mannheim) for biotinylated antibody detection. After two washes with PBS-0.05% Tween-20 color was developed by adding 2.2'-azino-di(-3-ethylbenzthiazolinesulfonate) (ABTS; Boehringer Mannheim) and 0.05% hydroxide peroxide, and the optical density was read at 405 nm.

3. Results

3.1. Binding of LIGHT to HveA (200t) and its N-terminal fragment HveA (120t)

HveA (120t), a truncated form of HveA (200t), consists of domains CRP-1 and CRP-2 and has recently been shown to bind viral gD with the same affinity as does HveA (200t) (Whitbeck et al., 2000). We first performed two ELISAs to determine whether LIGHT would also bind to these domains of HveA (Fig. 1). In the first assay, HveA (120t), HveA (200t) and a control protein, BSA, were immobilized on microtiter plates and incubated with various dilutions of LIGHT; binding of LIGHT was then detected with a monoclonal antibody. As shown in Fig. 1(A), LIGHT bound only to the full-length receptor ectodomain, HveA (200t). To establish that this result was not an artifact related to the coating of the proteins onto the plates, a second assay was then used. In this inhibition ELISA (Fig. 1(B)), LIGHT was preincubated with serial dilutions of HveA (120t) or HveA (200t) and then incubated with immobilized HveA (200t). In accordance with the direct binding assay HveA (120t) had no inhibitory effect on the binding of LIGHT to HveA (200t). These results

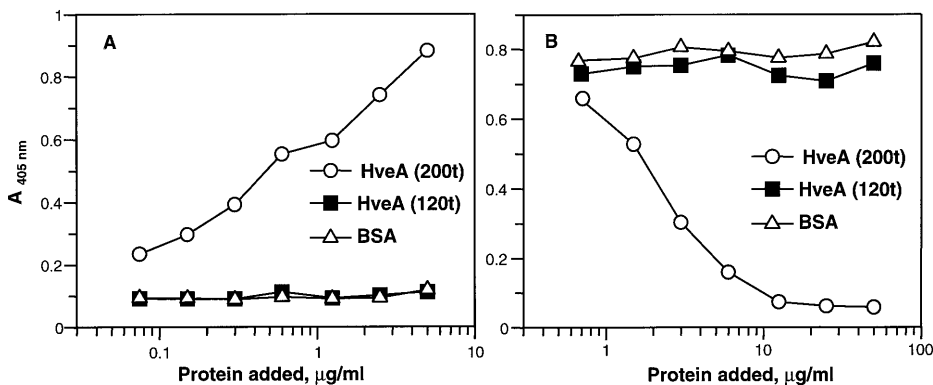


Fig. 1. Binding of LIGHT to HveA and its N-terminal fragment, HveA (120t). (A) Microtiter wells were coated with HveA (200t), HveA (120t) or control BSA and incubated with decreasing concentrations of LIGHT. (B) LIGHT was incubated with soluble HveA (200t), HveA (120t), or control BSA and then added to wells containing immobilized HveA (200t). LIGHT binding was detected with an anti-M2 flag monoclonal antibody, followed by a peroxidase-conjugated anti-mouse IgG antibody and ABTS peroxidase substrate.

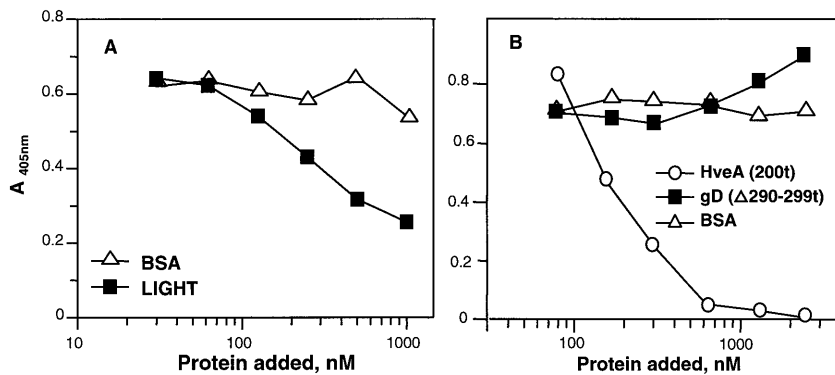


Fig. 2. Competition between gD and LIGHT for binding to HveA. HveA (200t)-coated plates were incubated with various concentrations of LIGHT or BSA (A) or gD (Δ 290-299t) or BSA (B). (A) A constant amount of gD (306t) was added to the plate, and the level of gD binding was detected with an anti-gD polyclonal antibody, followed by a peroxidase-conjugated anti-rabbit IgG antibody. (B) A constant amount of LIGHT was added to the plate, and its binding was detected with an anti-M2 flag monoclonal antibody, followed by a peroxidase-conjugated anti-mouse IgG antibody. In this assay, a polyclonal anti-gD antibody (R7) was added to parallel wells of the plate to ensure that the gD remained bound to HveA throughout the experiment (data not shown).

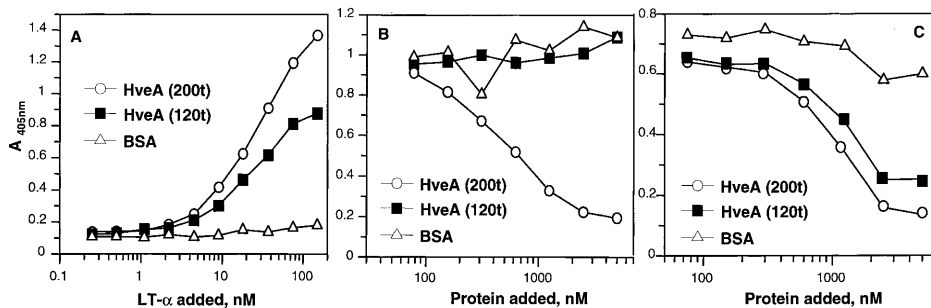


Fig. 3. Binding of LT- α to HveA (200t) and HveA (120t). (A) HveA (200t), HveA (120t) or BSA was immobilized on microtiter plates and incubated with various concentrations of LT- α . (B and C) LT- α was preincubated with different concentrations of HveA (200t), HveA (120t) or control BSA and then added to wells containing immobilized (B) HveA (200t) or (C) HveA (120t). Binding of LT- α was detected with an anti-LT- α monoclonal antibody (AG9) followed by a peroxidase-conjugated anti-mouse IgG antibody.

suggest that structural elements elsewhere in HveA (amino acids 120–200) are required for its interaction with LIGHT.

3.2. Competition between gD and LIGHT for binding to herpes virus entry mediator A

gD has been shown to bind to HveA (120t) and to compete HveA-Fc binding to receptor on the cell surface expressed LIGHT (Mauri et al., 1998). However, the results obtained in the previous experiment (Fig. 1) indicated that LIGHT does not bind to HveA (120t). This finding, therefore, raised the question of whether gD and LIGHT would compete for binding to HveA in an ELISA assay. To answer this question, we carried out a competition ELISA in which HveA (200t) was immobilized on the microtiter plate, and then incubated with various concentrations of LIGHT. Wild type gD, gD (306t) was then added, and its binding was detected with a polyclonal antibody. We indeed observed an inhibition of gD binding to HveA (200t) in the presence of LIGHT (Fig. 2(A)). To our surprise, however, when

we tested whether increasing amounts of gD (Δ 290-299t), a mutant form of gD with a 100-fold higher affinity for HveA than wild-type gD, would affect the interaction of LIGHT with immobilized HveA (200t), we observed no inhibitory effect, regardless of how much gD was added (Fig. 2(B)). These seemingly discordant results may reflect the affinity differences of LIGHT and gD for HveA, i.e., the affinities of the HveA-gD (306t) and HveA-gD (Δ 290-299t) interaction are 3.2 μ M and 30 nM, respectively (Willis et al., 1998), while that of LIGHT binding to HveA is 3.9 nM (Rooney et al., 2000).

3.3. Binding of lymphotoxin- α to HveA (200t) and HveA (120t)

To test whether LT- α would bind to the HveA (120t) protein, several ELISAs were carried out (Fig. 3). We first investigated whether LT- α would bind to HveA (120t) in a direct ELISA, by coating microtiter plates with HveA (200t), HveA (120t), or BSA and then adding various concentrations of LT- α . In this assay

(Fig. 3(A)), LT- α bound to both forms of the receptor. We also studied this interaction in solution: LT- α was preincubated with various concentrations of either HveA (200t), HveA (120t), or BSA and then added to plates coated with HveA (200t) (Fig. 3(B)) or HveA (120t) (Fig. 3(C)). The amount of LT- α bound was then detected with a monoclonal antibody. Fig. 3(B) shows that HveA (120t) was unable to compete with LT- α for binding to immobilized HveA (200t). However, HveA

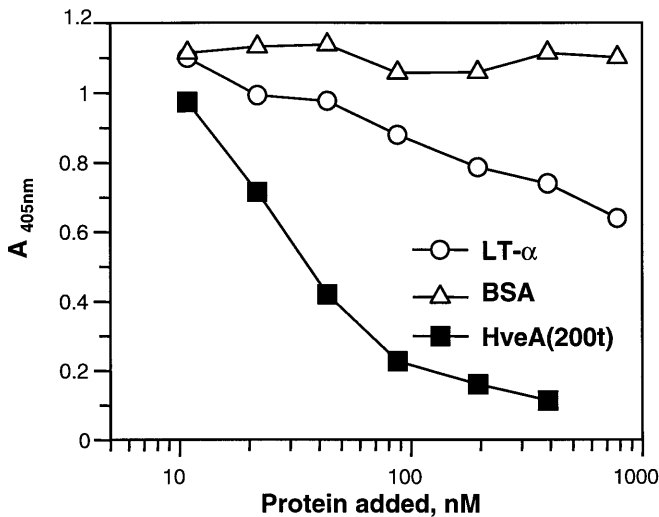


Fig. 4. Effect of LT- α on LIGHT binding to HveA (200t). LIGHT was preincubated with various concentrations of LT- α , HveA (200t) or BSA and then added to a microtiter plate on which HveA (200t) was immobilized. Bound LIGHT was detected with the M2 anti-FLAG monoclonal antibody followed by a peroxidase-conjugated anti-mouse IgG antibody.

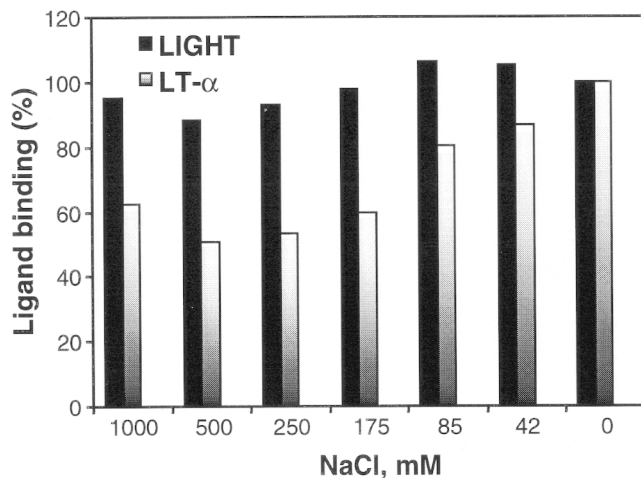


Fig. 5. Effect of salt concentration on LIGHT and LT- α binding to HveA. HveA (200t) was immobilized onto a microtiter plate, and constant amounts of LIGHT and LT- α were added in the presence of various concentrations of NaCl. Ligand binding was detected with an anti-flag M2 monoclonal antibody (for LIGHT detection) or an anti-LT- α monoclonal antibody (AG9), followed by a peroxidase-conjugated anti-mouse IgG antibody. The data represent the average of duplicate wells.

(200t) and HveA (120t) showed a relatively similar inhibitory effect on LT- α binding to HveA (120t). Taken together, these results suggest that LT- α is able to bind to the first two domains of HveA (CRP-1 and CRP-2), but with a lower affinity than to the intact HveA. Thus, it appears that structural elements in addition to those in HveA (120t) also participate directly or indirectly in the receptor's interaction with LT- α .

3.4. Lymphotoxin- α competes with LIGHT for binding to HveA (200t)

Since LT- α and LIGHT are homologous proteins (Mauri et al., 1998), we asked whether they would interact with HveA in a similar fashion and would therefore compete for binding to HveA. We performed an ELISA assay in which microtiter wells were coated with HveA (200t) and incubated with various concentrations of LT- α or BSA. LIGHT was then added to the plate, and its binding was detected with a monoclonal anti-FLAG M2 antibody. In this assay, LT- α was able to inhibit the binding of LIGHT to the receptor (Fig. 4), indicating that these proteins compete for interaction with HveA. However, the inhibitory effect of LT- α on LIGHT binding to HveA (200t) appeared to be partial, as compared to that observed for soluble HveA (200t).

3.5. Effect of salt concentration on the binding of lymphotoxin- α and LIGHT to HveA (200t)

We next examined whether the presence of increasing salt concentrations would affect the binding of HveA to its ligands. Incubation of LT- α and LIGHT with immobilized HveA (200t) was carried out in buffer supplemented with NaCl (0–1000 mM). Bound LT- α and LIGHT were detected with monoclonal anti-LT- α (AG9) and M2 anti-FLAG antibodies, respectively. Whereas, increasing salt concentrations had a significant effect on LT- α binding to HveA, the binding of LIGHT to HveA was relatively insensitive to salt concentration (Fig. 5). These data support the conclusion that the interaction of HveA with LIGHT differs from that of the receptor with LT- α .

3.6. Peptides BP-1 and BP-2 do not inhibit the binding of LIGHT to herpes virus entry mediator A

We have previously shown that the synthetic peptides BP-1 and BP-2 bind to HveA, and differentially inhibit binding of LT- α and gD to the receptor. We performed an ELISA assay to assess the effect of BP-1 and BP-2 on LIGHT binding to HveA. HveA (200t) was coated onto the wells of a microtiter plate and incubated with various concentrations of BP-1 and BP-2 or control

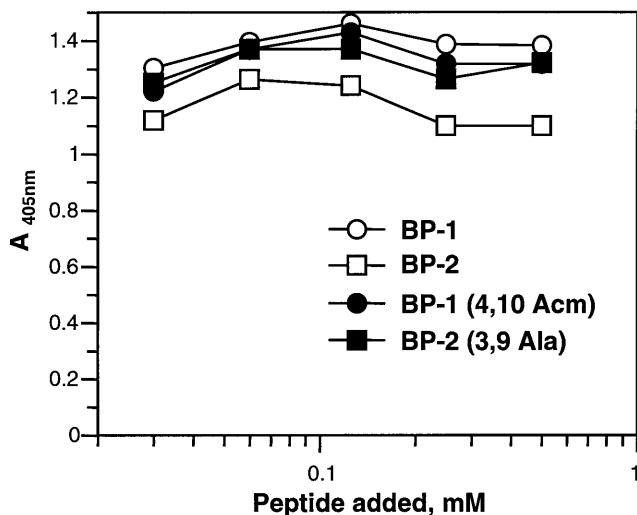


Fig. 6. Effect of peptides BP-1 and BP-2 on LIGHT binding to HveA. HveA (200t) was immobilized onto microtiter wells and incubated with various concentrations of peptides BP-1 and BP-2 or control peptides BP-1 (4,10 Acm) and BP-2 (3,9 Ala). LIGHT (A) or phage displaying BP-2 (B) were added, and their binding was detected with an anti-flag M2 monoclonal antibody, followed by a peroxidase-conjugated anti-mouse IgG antibody or a peroxidase-conjugated anti-M13 monoclonal antibody, respectively.

peptides BP-1 (4,10 Acm) and BP-2 (3,9 Ala) in the presence of 1.3 nM LIGHT, and the binding of LIGHT was then detected with a monoclonal antibody. Binding of LIGHT to HveA was not inhibited by either the BP-1 or BP-2 peptide (Fig. 6A). In contrast, we carried out a parallel assay in which the BP-1 and BP-2 peptides, but not their controls [BP-1 (4,10 Acm) and BP-2 (3,9 Ala)], were found to inhibit the binding of phage displaying BP-2 to HveA (200t), as previously reported (data not shown) (Sarrias et al., 1999). We also tested whether the binding of LIGHT to HveA would affect the binding of BP-1 and BP-2. For this purpose, we preincubated various concentrations of LIGHT with HveA immobilized on an ELISA plate, before the addition of

phage displaying either BP-1 or BP-2. We found that the same amount of either phage bound to HveA, regardless of the amount of LIGHT present in the well (data not shown).

3.7. Effect of anti-HveA monoclonal antibodies on ligand binding to herpes virus entry mediator A

A competition assay was performed to analyze the effect of six anti-HveA monoclonal antibodies on the binding of LIGHT and phage displaying BP-1 or BP-2 to HveA. HveA was immobilized on microtiter plates and incubated with various concentrations of each of the monoclonal antibodies. LIGHT or phage displaying BP-1 or BP-2 was then added, and their binding was detected with the addition of a biotinylated anti-flag M2 antibody followed by peroxidase-labeled streptavidin (for LIGHT detection), or a peroxidase-labeled anti-M13 antibody (for phage detection). Of the six antibodies, only CW3 was able to inhibit the binding of LIGHT and phage displaying BP-1 or BP-2 to HveA (Fig. 7(A–C)). This antibody has previously been found to bind to an epitope in HveA (120t), and inhibit the binding of viral gD to the receptor (Whitbeck et al., 2000).

3.8. Localization of the binding site of peptides BP-1 and BP-2 on herpes virus entry mediator A

To determine whether BP-1 and BP-2 would bind to the N-terminal fragment of HveA, HveA (120t), an ELISA assay was performed (Fig. 8), in which microtiter wells were coated with HveA (200t) or HveA (120t) and incubated with serial dilutions of phage supernatants displaying either BP-1 (Fig. 8A) or BP-2 (Fig. 8B). Neither phage displaying BP-1 nor BP-2 bound to HveA (120t), suggesting that HveA (120t) does not contain all the structural elements necessary for the interaction of the receptor with either peptide. This result also indicates that the binding site(s) of BP-1 and BP-2 on the receptor differ from that of gD.

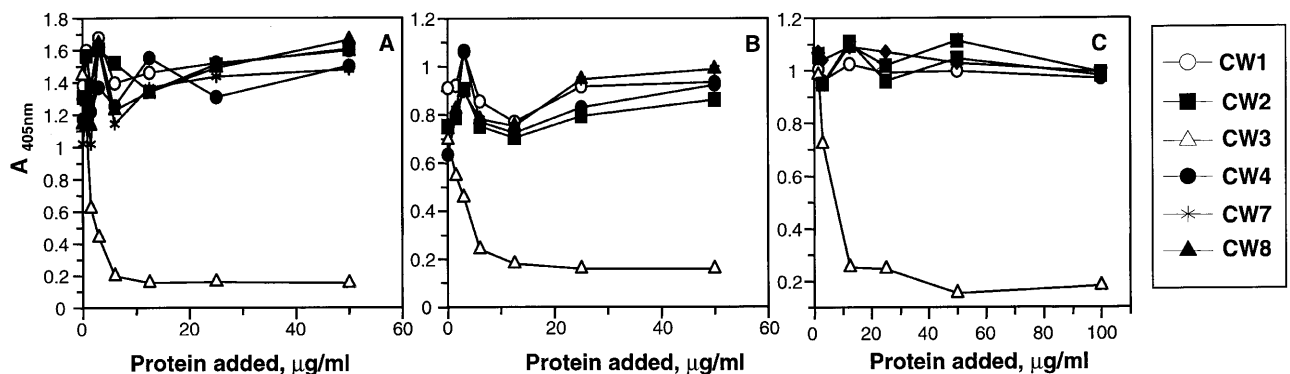


Fig. 7. Effect of anti-HveA monoclonal antibodies on ligand binding to HveA. HveA was immobilized on an ELISA plate and then incubated with various concentrations of one out of six anti-HveA monoclonal antibodies (CW1, CW2, CW3, CW4, CW7 and CW8). Phage displaying BP-1 (A), or BP-2 (B) or the protein LIGHT (C) were then added and their binding was detected with a peroxidase-conjugated anti-M13 monoclonal antibody, or a M2 anti-FLAG monoclonal antibody, followed by a peroxidase-conjugated anti-mouse IgG antibody.

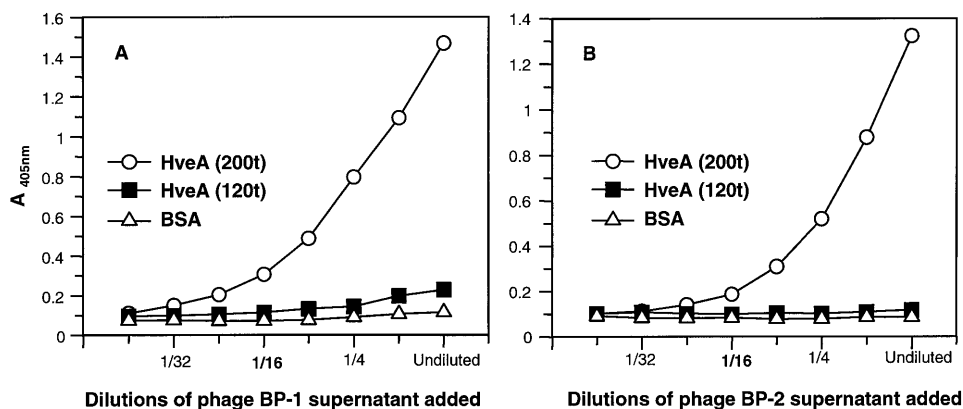


Fig. 8. Binding of BP-1 and BP-2 to HveA (200t) and HveA (120t). Microtiter wells were coated with HveA (200t), HveA (120t) or BSA and incubated with different amounts of phage displaying either (A) BP-1 or (B) BP-2. Bound phage was detected with a peroxidase-conjugated anti-M13 monoclonal antibody.

4. Discussion

In the present study, we have investigated the interactions of LT- α , LIGHT, gD, and two HveA-binding peptides with HveA, their common receptor. A better understanding of these interactions would shed light on the structural relationships and function of these proteins, some of which play key roles in the host immune response. In particular, we wondered whether LT- α and LIGHT interact with the same binding site on HveA.

Our results suggest that LIGHT and LT- α bind to overlapping sites on the receptor. However, the areas recognized by the two proteins are not identical, and the mode of their interaction apparently differs. We have analyzed their binding to HveA by using a panel of monoclonal antibodies directed against HveA, together with two HveA-binding peptides (BP-1 and BP-2) previously isolated from phage-displayed libraries (Sarrias et al., 1999). We also used a truncated recombinant form of the receptor, HveA (120t), which lacks the two C-terminal CRP domains (Whitbeck et al., 2000). Glycoprotein gD bound to this truncated molecule with the same affinity as it did to the full receptor ectodomain, indicating that the binding site of gD resides in the first two CRP domains of HveA (Whitbeck et al., 2000). This finding also suggests that the overall structure of HveA (120t) was not markedly altered as a result of CRP domain truncation.

Our direct binding assays and competition assays involving HveA (200t) and HveA (120t) indicated that LIGHT is not able to bind to HveA (120t) (Fig. 1). Because gD binds to this truncated portion of the receptor (Whitbeck et al., 2000), and LIGHT and gD have been shown to compete for binding to HveA (Mauri et al., 1998), it is necessary to consider the possibility that sequence truncation in HveA (120t) may have locally affected the conformation of the LIGHT

binding region on the receptor. Alternatively, binding of LIGHT to HveA may induce a conformational change in the receptor, thereby altering its ability to bind to gD. In contrast to LIGHT, LT- α was found to interact with the N-terminal portion of HveA [HveA (120t)] in a direct binding ELISA (Fig. 3). However, the truncated HveA (120t) failed to inhibit the interaction between LT- α and the full receptor ectodomain (HveA (200t)) (Fig. 3(B)), suggesting that structural elements other than those in HveA (120t) may also participate in the receptor's interaction with LT- α . In fact, it is possible that the LT- α -HveA interaction resembles that of LT- α with TNFR1: studies of co-crystallized LT- α and TNFR1 have revealed that LT- α binds to CRP domains two and three of TNFR1 (Banner et al., 1993).

Other data obtained in this study also support our conclusion that the interaction of LIGHT and LT- α with HveA differs. For example, we observed that although LT- α was able to inhibit the LIGHT-HveA interaction, this inhibition was partial, as compared to the much greater inhibitory effect of soluble HveA (200t) itself (Fig. 4). Moreover, when we investigated the effect of salt concentration on LT- α and LIGHT binding to the receptor, we observed significant differences between the two ligands (Fig. 5). The presence of high concentrations of NaCl (to 1 M) reduces the electrostatic interaction between proteins, without significantly affecting hydrogen bonds or van der Waals contacts (Dill, 1990; Blom et al., 2000). LT- α was susceptible to increasing amounts of salt in the binding buffer (Fig. 5), an indication that electrostatic interactions are involved in its binding to HveA. However, because we could not achieve a total suppression of LT- α binding to HveA even at 1 M NaCl, it is likely that other types of interactions also participate in its binding to the receptor. In contrast, the binding of LIGHT to HveA was not significantly disturbed by the presence of high amounts of salt, suggesting that the

LIGHT-HveA interaction may primarily involve hydrophobic contacts and hydrogen bonds.

The two HveA-binding peptides BP-1 and BP-2 used in this study were previously found to differentially inhibit the binding of LT- α and gD to the receptor (Sarrias et al., 1999). Our observation that they had no inhibitory effect on LIGHT binding to HveA (Fig. 6) is a further indication that the three HveA receptor ligands, gD, LT- α and LIGHT, bind to distinct sites on HveA. These results also indicated that BP-1 was exclusively able to affect the HveA-LT- α interaction. Because several TNFR family members that interact with LT- α can be expressed on the cell surface, it is often difficult to study the interaction between specific TNF proteins and their receptors. Therefore, BP-1 offers an ideal tool for investigating the cellular response to the LT- α -HveA interaction and its role in the host immune system.

To further characterize the HveA-ligand interactions, we used a panel of anti-HveA monoclonal antibodies and tested their ability to inhibit the binding of the HveA ligands to the receptor (Fig. 7). The binding site(s) of those mAbs to HveA, as mapped by Whitbeck et al. (Whitbeck et al., 2000), are as follows: CW1, CW2, CW3 and CW4 bind to the N-terminal region of HveA (HveA (120t)), whereas, CW7 and CW8 bind to the C-terminal domains CRP 3 and CRP 4 of the receptor. To our surprise, only mAb CW3 was able to inhibit the binding of BP-1, BP-2 and LIGHT to the receptor. It is remarkable that while the CW3 antibody bound to the truncated HveA (120t) neither LIGHT

nor BP-1 or BP-2 appeared to bind to this portion of the molecule (Figs. 1 and 8A and Fig. 8B, respectively). However, it is interesting that the same CW3 antibody has also been reported to be the only one capable of blocking the gD-HveA interaction (Whitbeck et al., 2000). Taken together, these data support our previously proposed model in which we hypothesized that HveA may undergo a conformational change upon ligand binding that may then affect its interaction with its other ligands.

Fig. 9 presents a model that summarizes our observations. We propose that LIGHT, LT- α and gD interact with different residues on HveA, although there may be some overlapping contact residues. The gD binding site on HveA has been localized to CRP domains one and two (Whitbeck et al., 2000). Based on our current results and on previous crystallographic data concerning the LT- α -TNFR1 interaction, we suggest that LIGHT and LT- α may interact with domains two and three of the receptor, yet rely on different modes of interaction. The data obtained with peptides BP-1 and BP-2 provide further support for our conclusions.

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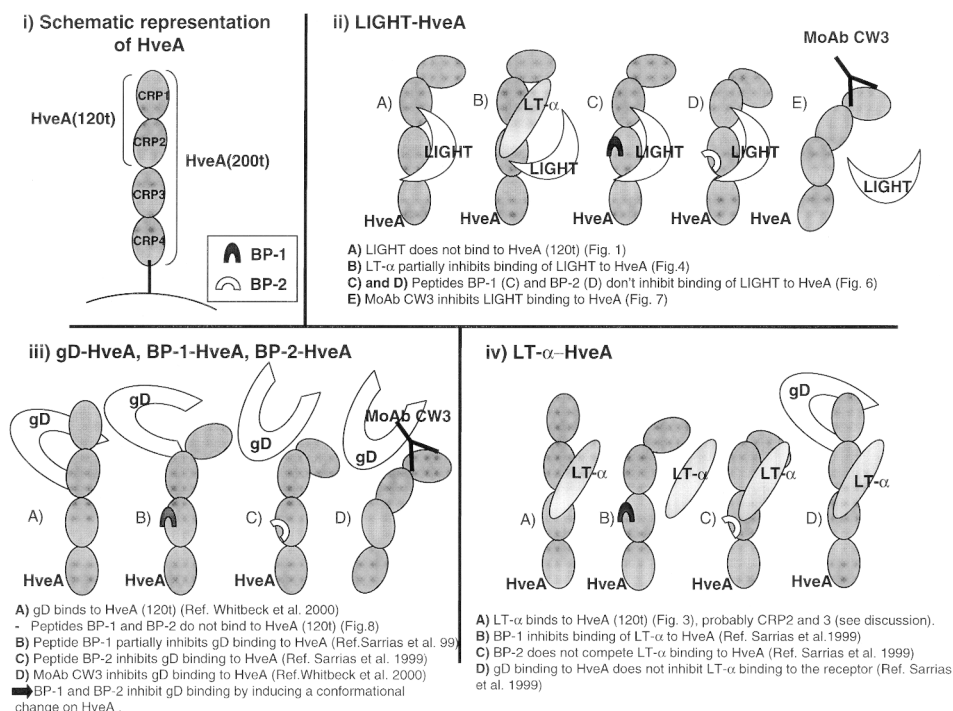


Fig. 9. Proposed model of the interaction between HveA and its ligands.

References

- Banner, D.W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.J., Broger, C., Loetscher, H., Lesslauer, W., 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* 73, 431–445.
- Blom, A.M., Berggard, K., Webb, J.H., Lindahl, G., Villoutreix, B.O., Dahlback, B., 2000. Human C4b-binding protein has overlapping, but not identical, binding sites for C4b and streptococcal M proteins. *J. Immunol.* 164, 5328–5336.
- Browning, J.L., Dugas, I., Ngamek, A., Bourdon, P.R., Ehrenfels, B.N., Miatkowski, K., Zafari, M., Yampaglia, A.M., Lawton, P., Meier, W., Benjamin, C.P., Hession, C., 1995. Characterization of surface lymphotoxin forms – use of specific monoclonal-antibodies and soluble receptors. *J. Immunol.* 154, 33–46.
- Crowe, P.D., Vanarsdale, T.L., Walter, B.N., Dahms, K.M., Ware, C.F., 1994. Production of lymphotoxin (LT-alpha) and a soluble dimeric form of its receptor using the baculovirus expression system. *J. Immunol. Meth.* 168, 79–89.
- Dill, K.A., 1990. Dominant forces in protein folding. *Biochemistry* 29, 7133–7155.
- Harrop, J.A., Reddy, M., Dede, K., Brigham-Burke, M., Lyn, S., Tan, K.B., Silverman, C., Eichman, C., DiPrinzio, R., Spanpanato, J., Porter, T., Holmes, S., Young, P.R., Truneh, A., 1998. Antibodies to TR2 (herpes virus entry mediator), a new member of the TNF receptor superfamily, block T cell proliferation, expression of activation markers, and production of cytokines. *J. Immunol.* 161, 1786–1794.
- Isola, V.J., Eisenberg, R.J., Siebert, G.R., Heilman, C.J., Wilcox, C., Cohen, G.H., 1989. Fine mapping of antigenic site-II of herpes-simplex virus glycoprotein-D. *J. Virol.* 63, 2325–2334.
- Kwon, B., Youn, B.S., Kwon, B.S., 1999. Functions of newly identified members of the tumor necrosis factor receptor/ligand super-families in lymphocytes. *Curr. Opin. Immunol.* 11, 340–345.
- Kwon, B.S., Tan, K.B., Ni, J., Oh, K.O., Lee, Z.H., Kim, K.K., Kim, Y.J., Wang, S., Gentz, R., Yu, G.L., Harrop, J., Lyn, S.D., Silverman, C., Porter, T.G., Truneh, A., Young, P.R., 1997. A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. *J. Biol. Chem.* 272, 14272–14276.
- Matsumoto, M., 1999. Role of TNF ligand and receptor family in the lymphoid organogenesis defined by gene targeting. *J. Med. Invest* 46, 141–150.
- Mauri, D.N., Ebner, R., Montgomery, R.I., Kochel, K.D., Cheung, T.C., Yu, G.L., Ruben, S., Murphy, M., Eisenberg, R.J., Cohen, G.H., Spear, P.G., Ware, C.F., 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpes virus entry mediator. *Immunity* 8, 21–30.
- Montgomery, R.I., Warner, M.S., Lum, B.J., Spear, P.G., 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87, 427–436.
- Moore, W.T., 1993. Integration of mass spectrometry into strategies for peptide synthesis. *Biol. Mass. Spectrom.* 22, 149–162.
- Moore, W.T., 1997. Laser desorption mass spectrometry. *Meth. Enzymol.* 289, 520–542.
- Nicola, A.V., de Leon, M.P., Xu, R.L., Hou, W.F., Whitbeck, J.C., Krummenacher, C., Montgomery, R.I., Spear, P.G., Eisenberg, R.J., Cohen, G.H., 1998. Monoclonal antibodies to distinct sites on herpes simplex virus (HSV) glycoprotein D block HSV binding to HVEM. *J. Virol.* 72, 3595–3601.
- Nicola, A.V., Willis, S.H., Naidoo, N.N., Eisenberg, R.J., Cohen, G.H., 1996. Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D. *J. Virol.* 70, 3815–3822.
- Raftery, M.J., Behrens, C.K., Muller, A., Krammer, P.H., Walczak, H., Schonrich, G., 1999. Herpes simplex virus type 1 infection of activated cytotoxic T cells: induction of fratricide as a mechanism of viral immune evasion. *J. Exp. Med.* 190, 1103–1114.
- Rooney, I.A., Butrovich, K.D., Glass, A.A., Borboroglu, S., Benedict, C.A., Whitbeck, J.C., Cohen, G.H., Eisenberg, R.J., Ware, C.F., 2000. The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J. Biol. Chem.* 275, 14307–14315.
- Sarrias, M.R., Whitbeck, J.C., Rooney, I.A., Spruce, L., Kay, B.K., Montgomery, R.I., Spear, P.G., Ware, C.F., Eisenberg, R.J., Cohen, G.H., Lambris, J.D., 1999. Inhibition of herpes simplex virus gD and lymphotoxin – a binding to HveA by peptide antagonists. *J. Virol.* 73, 5681–5687.
- Sisk, W.P., Bradley, J.D., Leopold, R.J., Stoltzfus, A.M., DeLeon, M.P., Hilf, M., Peng, C., Cohen, G.H., Eisenberg, R.J., 1994. High-level expression and purification of secreted forms of herpes-simplex virus type-1 glycoprotein GD synthesized by baculovirus-infected insect cells. *J. Virol.* 68, 766–775.
- Smith, C.A., Farrar, T., Goodwin, R.G., 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 76, 959–962.
- Whitbeck, J.C., Connolly, E.S., Willis, S.H., Hou, W., Krummenacher, C., Ponce de Leon, M., Lou, H., Baribaud, I., Eisenberg, R.J., Cohen, G.H., 2001 (Jan). Localization of the gD-binding region of the human HSV receptor. HveA. *J. Virol.*, in press.
- Whitbeck, J.C.P., Peng, G., Lou, H., Xu, R., Willis, S.H., Ponce de Leon, M., Peng, T., Nicola, A.V., Montgomery, R.I., Warner, M.S., Soulika, A.M., Spruce, L.A., Moore, W.T., Lambris, J.D., Spear, P.G., Cohen, G.H., Eisenberg, R.J., 1997. Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. *J. Virol.* 71 (8), 6083–6093.
- Willis, S.H., Rux, A.H., Peng, C., Whitbeck, J.C., Nicola, A.V., Lou, H., Hou, W.F., Salvador, L., Eisenberg, R.J., Cohen, G.H., 1998. Examination of the kinetics of herpes simplex virus glycoprotein D binding to the herpes virus entry mediator, using surface plasmon resonance. *J. Virol.* 72, 5937–5947.
- Zhai, Y., Guo, R., Hsu, T.L., Yu, G.L., Ni, J., Kwon, B.S., Jiang, G.W., Lu, J., Tan, J., Ugustus, M., Carter, K., Rojas, L., Zhu, F., Lincoln, C., Endress, G., Xing, L., Wang, S., Oh, K.O., Gentz, R., Ruben, S., Lippman, M.E., Hsieh, S.L., Yang, D., 1998. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J. Clin. Invest* 102, 1142–1151.