Interactions of HLA-B27 with the peptide loading complex as revealed by heavy chain mutations

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Abstract

MHC class I heavy chains assemble in the endoplasmic reticulum with β₂-microglobulin and peptide to form heterotrimers. Although full assembly is required for stable class I molecules to be expressed on the cell surface, class I alleles can differ significantly in their rates of, and dependencies on, full assembly. Furthermore, these differences can account for class I allele-specific disparities in antigen presentation to T cells. Recent studies suggest that class I assembly is assisted by an elaborate complex of proteins in the endoplasmic reticulum, collectively referred to as the peptide loading complex. In this report we take a mutagenesis approach to define how HLA-B27 molecules interact with the peptide loading complex. Our results define subtle differences between how B27 mutants interact with tapasin (TPN) and calreticulin (CRT) in comparison to similar mutations in other mouse and human class I molecules. Furthermore, these disparate interactions seen among class I molecules allow us to propose a spatial model by which all class I molecules interact with TPN and CRT, two molecular chaperones implicated in facilitating the binding of high-affinity peptide ligands.

Introduction

There is a striking association between the expression of HLA-B27 molecules and susceptibility to a group of closely related arthritic diseases collectively referred to as spondyloarthropathies (1). Although this association suggests direct involvement of HLA-B27 in disease pathogenesis, the mechanism remains unclear in spite of intense investigation (2–5). Most of these investigations have focused on identifying unique arthritogenic peptides that bind to HLA-B27 (6). The current dogma is that T cell responses to putative arthritogenic peptides may be initially induced by infection with Gram-negative bacteria such as Klebsiella, Chlamydia, Shigella or Yersinia (1). Subsequently, it has been proposed that these B27-restricted T cells are autoreactive such that they detect peptides derived from normal tissues such as the joint. Although this attractive model is consistent with considerable published evidence, arthritogenic peptides detected by B27-restricted T cells have not been identified. What is perhaps most surprising, is the failure to identify disease-related B27 peptide ligands in rodent models that have transgenic expression of HLA-B27 molecules and are susceptible to arthritic disease (3–5).

The failure to identify arthritic peptide ligands of B27 has resulted in more recent studies proposing that B27 molecules may have disease-related properties other than specific peptide binding. For example, Peh et al. (7) reported that B27 molecules are less dependent upon the chaperone tapasin (TPN) than other class I molecules. Poor interaction with TPN could result in poor peptide loading in the endoplasmic reticulum (ER) and subsequently a greater propensity for B27 to bind peptides at the cell surface. Alternatively, Mear et al. (8) reported that the unique B pocket architecture results in the misfolding and premature turnover of a portion of B27 molecules, by an unknown mechanism that could involve disparate chaperone interaction. Additionally, Allen et al. (9) reported that B27 heavy chains can be expressed as novel β₂-microglobulin (β₂m)-free heavy chain homodimers
that have unique antigen presentation properties. In a perhaps related study, Khare et al. (10) reported that in a murine HLA-B27 transgenic model, β2m-deficient mice develop arthritis and that disease can be blocked with mAb to free B27 heavy chains. These combined findings underscore the importance of defining the precise mechanism of B27 assembly and folding.

Although allele differences have been noted (11), prior to peptide binding most class I molecules are in physical association with the complex of transporter associated with antigen processing (TAP) (12–14), TPN (15–17), calreticulin (CRT) (15,17,18) and ERp57 (19–21). This complex, collectively referred to here as the peptide loading complex, has been implicated in facilitating class I folding and assembly by unknown mechanisms (22). Furthermore, the contribution of individual members of the peptide loading complex remains undefined, as does the manner in which they spatially associate with the class I molecule. To address these questions, we and others have shown that mutations at three sites in the class I H chain determine its association with TAP, TPN and CRT (14,23–28). Interestingly, each of these three sites is located in each of the three class I extracellular domains, α1, α2 and α3. However, due to an apparent requirement for cooperative binding (28,29), it has been difficult to unequivocally determine which chaperone interacts with which of the three sites on the class I H chain.

In this study we introduced mutations into the B27 heavy chain at the three sites previously implicated in ER chaperone association. Our findings define unique properties of B27 in regard to ER chaperone associations, and strongly support a general model of how all class I molecules spatially associate with TPN and CRT.

Methods

mAb and antisera

The mAb 64-3-7 (IgG2b) is specific for open forms of Ld/Ld and other molecules tagged with this epitope (30–32). mAb ME-1 (IgG1) recognizes assembled HLA-B27 H chains (33) and mAb BBM1 (IgG2b) recognizes human β2m (34). The anti-human TAP antibody (#1478) (25) and the anti-human TPN antibody (#1848) were generated in rabbits immunized with peptides derived from the N-terminal sequence of the respective human proteins. Anti-CRT antisera were purchased from either Stressgen (SPA-600, rabbit antibody; Victoria, BC, Canada) or Affinity Bioreagents (PA1-903, chicken antibody; Golden, CO).

Cell lines and flow cytometry

HeLa cells were maintained at 37°C in complete medium containing 10% bovine calf serum (Hyclone, Logan, UT). To analyze cells for surface expression of class I molecules, cells were stained with 20 μl of culture supernatant from 64-3-7 or ME-1 hybridomas by standard methods. The data collected on a FACScan (Becton Dickinson, San Jose, CA) were expressed in the form of histograms as fluorescence values (x-axis) plotted against cell numbers (y-axis) using CellQuest Software (Becton Dickinson).

Results and discussion

Approach

To detect its specific association with the peptide loading complex, we tagged the HLA-B27 molecule with the 64-3-7 epitope by introducing a single mutation (R48Q). The 64-3-7 epitope is only detected when H chains are in an open

Mutagenesis and transfection

The HLA-B*2705 cDNA was kindly provided by Dr Joel Taurog (University of Texas Southwestern Medical Center at Dallas) and it was subcloned in the mammalian expression vector RSV.5.neo (35). All mutageneses were performed using the Quik Change Mutagenesis kit from Stratagene (San Diego, CA) as previously described (32) with the exception of the S132K mutant which was constructed in an identical fashion to that of the Ld loop mutants (28). The mutants were named by first indicating the wild-type residue followed by the amino acid position in the mature protein and then the mutated residue. The HeLa cells were transfected with Lipo-lectin (Life Technologies, Gaithersburg, MD) according to a modified protocol based on the manufacturer’s instructions. The transfectants were selected in 0.6 mg/ml of active G418. HLA-B27 R48Q (etB27) molecules were transfected into the human B lymphoblastoid cell lines LCL721.221 and .220 (36) (kindly provided by Dr Thomas Spies, Fred Hutchinson Cancer Center, Seattle, WA) by electroporation using the Gene Pulser II system from BioRad (Hercules, CA).

Immunoprecipitations and Western blots

HeLa cells and HeLa transfectants were lysed in buffers containing 1% digitonin (Wako, Richmond, VA) in 10 mM Tris-buffered saline pH 7.4 (TBS) with 20 mM iodoacetamide and 0.2 mM of freshly added PSMF. Saturating amounts of the primary antibody were added to the lysis buffer and subjected to immunoprecipitations as previously described (25). Briefly, lysates were centrifuged to remove cell debris and nuclei and supernatants were added to Protein A–Sepharose CL-4B (Amersham Pharmacia, Uppsala, Sweden) for 60 min on ice and Protein A-bound material was washed in 0.1% digitonin in TBS. Immunoprecipitates were eluted from Protein A by boiling for 5 min in elution buffer consisting of 0.125 M Tris, pH 6.8, 2% SDS, 12% glycerol, 2% bromophenol blue (w/v). Samples were electrophoresed on either Tris–glycine or Tris–acetate, pre-poured gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). After overnight blocking in 10% milk, PBS/0.05% Tween 20, membranes were incubated in a dilution of antibody for 2 h, washed 3 times with PBS/0.05% Tween 20 and incubated for 1 h with biotin-conjugated goat anti-mouse IgG or anti-rabbit IgG (Caltag, San Francisco, CA) or rabbit anti-chicken/turkey IgG (Zymed, San Francisco, CA). Following three washes with PBS/0.05% Tween 20, membranes were incubated for 1 h with streptavi-din-conjugated horseradish peroxidase (Zymed, San Francisco, CA), washed 3 times with PBS/0.3% Tween 20 and incubated with ECL chemiluminescent reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were then developed on BioMax film (Eastman Kodak, Rochester, NY).
conformation and this epitope is not sterically blocked when class I is associated with the peptide loading complex (32). The 64-3-7 epitope is naturally present in only mouse L\textsuperscript{d} and L\textsuperscript{a} molecules, but has been successfully transferred into mouse K\textsuperscript{d}, K\textsuperscript{b}, M3 and Qa\textsuperscript{1b} molecules with either one or two amino acid substitutions (32,37,38). Furthermore, epitope transfer has been found not to alter the peptide binding signature of each of these class I molecules. Thus, by introducing this epitope into B27 we could quantify the peptide loading of B27 molecules by determining the percent of B27 molecules expressed in an open versus folded conformation and then compare these findings to those obtained with other epitope-tagged class I molecules. Furthermore, tagged B27 molecules allowed us to compare how B27 interacts with components of the peptide loading complex, relative to other class I molecules.

As shown in Fig. 1(c), expression of epitope-tagged B27 (etB27) in human HeLa cells rendered a subset of surface B27 molecules detectable with mAb 64-3-7. In studies of other class I molecules, 64-3-7\textsuperscript{+} conformers at the cell surface were shown to arise after peptide dissociation (31,38). As expected, endogenous class I molecules expressed by HeLa cells and wild-type B27 molecules are not 64-3-7\textsuperscript{+} (Fig. 1a and b respectively), demonstrating that mAb 64-3-7 uniquely detects etB27 molecules in HeLa cell transfectants. Thus, this is a valid test system to specifically study the assembly of B27 molecules. Relative to the amount of fully assembled etB27 (ME1\textsuperscript{+}) (33) there were relatively few open etB27 (64-3-7\textsuperscript{+}). Indeed, on the surface of HeLa-etB27 cells, only 4\% of etB27 molecules were detected in a open conformation. Similarly, on .221-etB27 cells the percent open was found to be 3\% (not shown). By comparison, the percentage of surface open forms is 7\% for K\textsuperscript{d}, 15\% for K\textsuperscript{b} and 28\% for L\textsuperscript{d} as observed on L cells or .221 cells (37). Furthermore, in data not shown the surface half-life of etB27 [like wild-type B27 (39)] was found to be >20 h, compared to reported half-lives of 2 and 10 h for L\textsuperscript{d} and K\textsuperscript{b} respectively (31 and unpublished data). These comparisons suggest that the overall quality of peptide loading of B27 is relatively good compared to these mouse cell I molecules.

**Importance of the N-linked glycan in the α1 domain**

To address which regions of B27 are required for chaperone interactions in the ER, mutations were introduced into etB27 molecules at sites in the α1, α2 and α3 domains previously implicated in ER chaperone association with other class I molecules (14,23–28). Earlier studies using castanospermine (18) demonstrated that an N-linked oligosaccharide is involved in class I association with TAP/TPN/CRT, the class I peptide loading complex. Furthermore, the location of the N-linked glycan at N86 in the α1 domain is of critical importance, since mutation of the glycosylation site N176 residue in the α2 domain of a mouse class I molecule did not affect its ability to associate with the peptide loading complex (25). To determine the importance of the N86 attached glycan of HLA-B27 for association with the peptide loading complex, etB27 N86Q molecules were expressed in HeLa cells. Although etB27 N86Q molecules were expressed at the surface of HeLa cells, the quality of peptide loading was clearly impaired compared with etB27. As shown in Fig. 1(B), 25\% of surface etB27 N86Q molecules were detected in a peptide-empty form (64-3-7\textsuperscript{+}), whereas only 4\% of etB27 molecules were detected in a peptide-empty form. As further evidence for peptide loading deficiencies, surface B27 N86Q molecules were found to be markedly inducible when cells were cultured at 25°C (data not shown), a hallmark of sub-optimal peptide loading (40). As shown in Fig. 2(A), the amount of β\textsubscript{2}m assembly was clearly impaired in N86Q compared with etB27 molecules. Even though the HeLa-etB27 and HeLa-etB27 N86Q cells expressed similar amounts of B27 H chain, the relative amount of N86Q H chain assembled with β\textsubscript{2}m was significantly reduced. This reduced assembly was seen when precipitating β\textsubscript{2}m and blutting for H chain with either mAb 64-3-7 (Fig. 2A) or when precipitating H chains with either mAb 64-3-7 or ME1 and blutting with
Fig. 2. Effects of individual etB27 mutation(s) on chaperone association. Lysates of HeLa cells transfected with the construct indicated on the top of the figure were immunoprecipitated and tested by Western blot analysis. (A) Respective lysates were immunoprecipitated with the reagent listed along the left side of the figure and each precipitate was then Western blotted with mAb 64-3-7 to specifically detect etB27 H chains. (B) Respective lysates were immunoprecipitated with 64-3-7 and then Western blotted to detect the associated ER protein listed along the left side of the figure. (C) Respective lysates were precipitated with the reagent listed along the left of the figure and then Western blotted with mAb BBM-1 to detect human β2m molecules. Antibodies used for this experiment were: anti-epitope tagged H chain (64-3-7), anti-BBM1 (SPA-600, StressGen Biotechnologies) and anti-CRT for (A) (SPA-600, StressGen Biotechnologies) and anti-CRT for (B) (PA1-903, Affinity BioReagents).

Fig. 3. Impaired peptide-induced folding of N86Q mutant B27 molecules compared to wild-type. Samples representing cell equivalents of etB27 or etB27 N86Q were lysed in 1% digitonin and incubated on ice for 2h with 200μM of a length-matched, non-B27 binding, control peptide (D12um-GPHSNFFGY) (42), no peptide or 200μM of B27 ligand HIV-gag (KRWIILGLNK) (41) as indicated along the top of the figure. Samples in (A) were precipitated with mAb 64-3-7 and the co-precipitating β2m molecules were detected by immunoblotting with mAb BBM-1. Results in (A) demonstrate that peptide can specifically induce the loss of β2m-associated open forms of wild-type B27 molecules, but not N86Q mutant molecules. Samples in (B) were precipitated with mAb ME-1 and the etB27 H chains were detected by blotting with mAb 64-3-7. Results in (B) demonstrate that peptide can specifically induce the gain of folded wild-type B27 H chains, but not N86Q mutant B27 H chains.

To determine if suboptimal assembly was indicative of aberrant associations with the peptide loading complex, etB27 and etB27 N86Q molecules were compared with regard to their associations with TAP, TPN and CRT. As shown in Fig. 2, lack of carbohydrate in the α1 domain resulted in a loss of association of etB27 molecules with TAP, TPN and CRT. Indeed, lack of association of etB27 N86Q with TAP/TPN/CRT was demonstrated by either precipitating etB27 H chains and blotting with antibody to the ER protein (Fig. 2B), or precipitating with antibody to the ER protein and blotting with mAb 64-3-7 to detect etB27 H chains (Fig. 2A). Thus like other mouse and human class I molecules studied so far, B27 is dependent upon N-linked glycosylation in the α1 domain for association with the assembly complex of TAP, TPN and CRT. Since CRT has been shown in other systems to have lectin-like activity, it seems likely that CRT specifically binds class I H chains via their α1 domain glycan (18). Failure to detect TAP and TPN in association with H chains carrying a mutation at N86 could be explained by CRT being a prerequisite for H chains to form stable complexes with TAP/TPN or by improper folding of class I molecules in the absence of carbohydrate addition.

To further characterize the impaired assembly of mutant N86Q H chains, peptides were added to lysates of HeLa-etB27 and HeLa-etB27 N86Q cells. For this experiment the HIV gag peptide (41) was used as a known B27 binder and the D12 tum- peptide (42) as a length-matched, control non-B27 binder. Incubation with the HIV peptide induced both the specific loss of β2m-associated open forms of etB27 (Fig. 3A, left panel) and the specific gain of assembled etB27 molecules as detected with ME1 (Fig. 3B, left panel). By contrast no peptide-specific conversion was observed with mutant N86Q molecules (Fig. 3A and B, right panel). Thus, these findings demonstrate that the N86Q mutation clearly impairs peptide-induced H chain folding in cell lysates, an
observation consistent with the role of the peptide loading complex in facilitating peptide binding.

Importance of residues around D227 in the α3 domain
Previous studies of mouse L^d (14), D^b (26) and D^d (27) molecules showed that mutations in the α3 domain around residue 227 disrupted their ability to associate with TAP, TPN and CRT. This residue is located on an exposed bridge that transects the α3 domain (Fig. 4). It is noteworthy that surface expression of L^d and D^d molecules was not appreciably reduced by single substitutions in the α3 domain (14,27). Furthermore, the double mutation of D227K and E229K of L^d was found to more completely disrupt association with TAP, TPN and CRT (43). Thus, to determine the role of this α3 site on B27 surface expression, etB27 D227K,E229K mutant molecules were expressed in HeLa cells. As shown in Fig. 1(f), etB27 molecules with the α3 mutations were expressed at a high level on the cell surface. Furthermore, the level of assembly with β2m was very similar between etB27 D227K,E229K versus etB27 molecules (Fig. 2A). However, etB27 molecules with the α3 mutation had a higher percentage of peptide empty forms at the cell surface (22%) compared with etB27 molecules without the α3 mutation (4%) (shown in Fig. 1c and f). Furthermore, surface etB27 D227K,E229K molecules were found to be more accessible to exogenous peptide ligands (data not shown). Thus the expression defect of this α3 mutant appears to involve impaired peptide loading.
To determine whether this impaired peptide loading was indicative of aberrant association with the peptide loading complex, etB27 D227K,E229K molecules were tested for their associations with TAP, TPN and CRT. As shown in reciprocal assays in Fig. 2(A and B), etB27 D227K,E229K molecules were found not to be associated with TAP, but were associated with CRT. Interestingly, in certain assays (e.g. Fig. 2A) etB27 D227K,E229K molecules were detected in association with TPN, whereas in other assays (e.g. Fig. 2B) no TPN association was detected. Our interpretation of these ambivalent findings is that this α3 mutation reduces association with TPN to near the threshold for detection in this assay. It is also important to note that this apparent weak association of etB27 D227K,E229K with TPN is not sufficient to promote its concomitant association with TAP. This finding suggests that class I molecules may have to achieve stable association with TPN via the α3 domain, for class I/TPN complexes to maintain association with TAP. This is an attractive model because it fits with studies of truncated forms of TPN demonstrating that membrane proximal regions of TPN determine its association with TAP (29). Furthermore, this model is consistent with results obtained with HLA-B44 and its disparate interactions with human versus mouse TPN (44). In any case, these findings with the etB27 D227K,E229K extend to a human class I molecule the importance of this site in the α3 domain for TAP/TPN association. Furthermore, the fact that α3 mutation of B27 can disrupt TPN association and not CRT association, supports the prediction that the α3 domain is a TPN interaction site (27).

**Importance of residues around position 132 in the α2 domain**

Initial evidence that a site in the α2 domain of the class I heavy chain determines its interaction with TAP/TPN/CRT came from studies of a human A2 molecule with an induced T134K mutation (23,24). Subsequent studies by us with the mouse Ld2 molecule showed that single substitutions in an extensive region of the α2 domain (residues 128–136) prevented its association with TAP/TPN/CRT (28). As shown in Fig. 4, this site is on a loop connecting a β strand that next assumes a helical conformation. Furthermore, it should be noted that both A2 T134K (23,24) and Ld T132K (28) extend to a human class I molecule the importance of this site in the α3 domain for TAP/TPN association. Furthermore, the fact that α3 mutation of B27 can disrupt TPN association and not CRT association, supports the prediction that the α3 domain is a TPN interaction site (27).

**T134K (23,24) and Ld2 mutants (28). Surprisingly, however, the S132K mutation did not affect the association of etB27 with CRT. This finding contrasts with earlier studies of A2 (45) and Ld2 (28) that demonstrated that mutations around 132–134 disrupt heavy chain association with CRT as well as TAP/tapasin. Thus B27, unlike A2 and Ld2, can proficiently form stable complexes with CRT without simultaneously binding TAP/TPN. Therefore, there appear to be differences among class I molecules in regard to their dependency on CRT to maintain stable association with TPN/TAP. Furthermore, our findings with B27 indicate that the α2 residues 128–136 are not involved in H chain interaction with CRT. Therefore, H chain residues 128–136 are likely a TPN interaction site.

**The initial association of CRT with HLA-B27 is dependent upon TPN**

The above findings with α2 and α3 mutants indicate that CRT can associate with class I in the absence of TPN. This finding was unexpected, since studies of several other mouse and human class I molecules have demonstrated that CRT and TPN required cooperative binding to maintain high levels of steady state association with class I (28,29,41). However, reports by us and others suggest that limited mutagenesis may not totally ablate H chain interaction with TPN/TAP (26,40). Thus, to more rigorously test the TPN-dependency of CRT binding to class I, associations were compared in lysates of TPN-deficient .220-etB27 cells and TPN-positive .221-etB27 cells. As shown in Fig. 5, CRT was not detected in association with etB27 in .220 cells. Thus CRT association requires TPN to form stable complexes with B27. The discordance between the mutagenesis data and the .220-etB27 data is intriguing. Perhaps the weak interaction between mutant H chains and TPN is sufficient to allow CRT to join the complex. It is also possible that the relatively unique C67 residue in the B pocket of B27 is unpaired, and forms an abnormal disulfide bonded intermediate that retains CRT interaction in the lysate and not TPN interaction. In any case, our data show that the continuous presence of TPN is not required for CRT to remain bound to class I H chain. A speculative model consistent with these data, and recent reports of others, is that TPN is required for the initial binding of a suboptimal peptide used to maintain the integrity of the ligand binding complex against the peptide loading complex.
site until a high-affinity peptide is available for binding (45). It could then be proposed that CRT can maintain interaction with the class I/low-affinity peptide complex in the absence of stable TPN interaction. Regardless of the validity of this model, the data presented here with etB27 provide additional evidence that H chain mutations are leaky in their interaction with members of the peptide loading complex. Thus given the cooperative nature of the association of CRT and TPN with class I, mutations are clearly advantageous for defining specific sites of interaction.

In summary, we show here that B27 molecules can be tagged with a novel epitope specific for their open conformation. Furthermore, using this approach we tested B27 molecules to determine whether they interact with the various components of the peptide loading complex in the ER in a manner similar to other human and mouse class I alleles. Like other mouse and human class I alleles studied thus far, HLA-B27 molecules appear to use at least three sites, one in each of the extracellular domains (α1, α2 and α3), to interact with components of the peptide loading complex (Fig. 4). Based on these findings with HLA-B27, we propose that TPN interacts with the α2 and α3 sites of the class I H chain, and that the α3 site may be more important for TPN-mediated TAP association. By contrast, the α2 site may be important for TPN monitoring an H chain conformational change when peptide binds. Indeed, a homologous loop on the opposing side of the H chain in the α1 domain is known to change conformation when peptide binds (32). Furthermore, the peptide induced change in the α2 site (residues 128–136) could be related to how the peptide specifically interacts with the F pocket of the peptide binding groove of the H chain as proposed by Tim Elliot (46). In this model the introduction of the C-terminal end of the peptide into the F pocket is accompanied by formation of hydrogen bonds that require the displacement of the α2 helix toward the cleft. This displacement could potentially alter the conformation of residues 128–136 that in turn induce H chain release from TPN. This model could explain why mutations or polymorphisms of HLA residues 115 and 116 (that are in or near the F pocket), have been found to influence class I association with the peptide loading complex (11,47,48). Based on additional findings reported here, we propose that CRT interacts via the α1 H chain glycan and not the α2 or α3 sites, and that CRT interaction with the H chain α1 glycan is TPN dependent initially, but may not require continuous TPN. From a spatial standpoint this is an attractive model, because the α2 and α3 sites are located on a common plane of the class I H chain (Fig. 4). Furthermore, residue N86 is spatially removed from the common plane shared by the α2 and α3 sites, thus allowing TPN and CRT to bind simultaneously to the class I heavy chain. Importantly, in this model both CRT and TPN are bound close to the peptide binding site of class I, whereby they could facilitate the binding of high-affinity ligands and/or monitor the release of class I from the loading complex once a high-affinity peptide has bound.

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Abbreviations

β2m β2-microglobulin
CRT calreticulin
CNX calnexin
ER endoplasmic reticulum
etB27 epitope-tagged B27
TAP transporter associated with antigen processing
TPN tapasin

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