The Host-Encoded Heme Regulated Inhibitor (HRI) Facilitates Virulence-Associated Activities of Bacterial Pathogens

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Abstract

Here we show that cells lacking the heme-regulated inhibitor (HRI) are highly resistant to infection by bacterial pathogens. By examining the infection process in wild-type and HRI null cells, we found that HRI is required for pathogens to execute their virulence-associated cellular activities. Specifically, unlike wild-type cells, HRI null cells infected with the gram-negative bacterial pathogen *Yersinia* are essentially impervious to the cytoskeleton-damaging effects of the Yop virulence factors. This effect is due to reduced functioning of the *Yersinia* type 3 secretion (T3S) system which injects virulence factors directly into the host cell cytosol. Reduced T3S activity is also observed in HRI null cells infected with the bacterial pathogen *Chlamydia* which results in a dramatic reduction in its intracellular proliferation. We go on to show that a HRI-mediated process plays a central role in the cellular infection cycle of the Gram-positive pathogen *Listeria*. For this pathogen, HRI is required for the post-invasion trafficking of the bacterium to the infected host cytosol. Thus by depriving *Listeria* of its intracellular niche, there is a highly reduced proliferation of *Listeria* in HRI null cells. We provide evidence that these infection-associated functions of HRI (an eIF2 α kinase) are independent of its activity as a regulator of protein synthesis. This is the first report of a host factor whose absence interferes with the function of T3S secretion and cytosolic access by pathogens and makes HRI an excellent target for inhibitors due to its broad virulence-associated activities.

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Introduction

Greater knowledge of the mechanisms employed by microbial pathogens to overcome host defenses has allowed for the development of drug-like molecules that specifically target these pathogen virulence-associated structures. Since at least some of these virulence-associated structures are widely conserved among pathogens of animals and plants, such ‘virulence block’ compounds provide an attractive alternative to conventional antibiotics that typically target structures (e.g., ribosomes) or processes (e.g., cell wall synthesis) found in both pathogens as well as members of the microbiota. Several examples of broad-acting small molecule virulence blockers were originally identified as inhibitors of the type 3 secretion (T3S) system of the pathogenic *yersinia* which delivers virulence factors directly into the host cell cytosol [1]. Subsequently it was shown that these compounds also inhibit T3SSs of other Gram-negative pathogens such as *Chlamydia, Salmonella*, and *Pseudomonas* [2]. Here we broaden this concept by identifying a host-encoded factor that is required by diverse pathogens to execute their respective cellular infection cycles.

In a yeast-based genetic screen using bacterial virulence factors as probes, we found that the stress-induced eIF2 signaling pathway plays a key role in the intracellular activities of both the *Yersinia* protein kinase A (YpkA) and *Yersinia* outer protein J (YopJ) [3]. In eukaryotes eIF2 signaling mediates the cellular responses to a variety of external and internal stress. Mammalian cells possess four different eIF2α kinases (GCN4, PERK, PKR, and HRI) that are activated by distinct stress
Infections

Transcription analysis was performed as previously described [4]. 

Materials and Methods

Host and pathogen strains

The heme-regulated inhibitor (HRI in humans, Hri in mice) was originally identified as the translation-level regulator (through its eIF2α kinase activity) that couples β-globin synthesis with heme levels during erythropoiesis and has more recently been shown to mitigate oxidative stress during erythropoiesis [5,6]. HRI is also important for various stress responses in yeast and mammalian cells [7,8]. Here we investigated whether HRI plays a role in host cell infection by microbial pathogens. Unexpectedly, we found that HRI positively regulates specific virulence-related activities of diverse bacterial pathogens. Surprisingly, these HRI effects were independent of its canonical function as a translation regulator via eIF2α and thus identify a novel role for HRI in bacterial pathogenesis.

Flow cytometry and imaging

For the antigen presentation and cytokine expression analyses, single-cell suspensions of splenocytes were prepared from Hri+/+ and -/- aged/match mice, passed through a 70 micron filter and resuspended in serum-containing media. Cells were restimulated for 1 hr and then infected with either the OVA-expressing L. monocytogenes strain or the Y. pseudotuberculosis strain. For the OVA experiments, splenocytes were infected for 5 h in the presence of brefeldin A and then stained with anti-CD11c (eBioscience), anti-B220 (BD Biosciences), anti-MHC I Ova (Biolengend), anti-CD11b (BD Biosciences), anti-CD4 (eBioscience), anti-CD8 (Biolengend), F4/80 (Caltag Laboratories), and Live/dead near IR (Invitrogen) in fluorescence-activated cell sorting buffer (PBS containing 2% bovine serum albumin and 0.1% sodium azide) for 30 min at 4 °C and then fixed in 2% paraformaldehyde. A Cytofix/ Cytoperm kit (BD-Pharmingen) was used to measure intracellular levels of TNFα. Data were acquired using a BD
FACS LSRII flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc.).

The microscopic-based *L. monocytogenes* infection assays were performed using techniques described above with the additional feature that the GFP-expressing bacteria were directly visualized. For the *L. monocytogenes* proliferation assay, MEFs were seeded in a 48-well plate (10⁵ per well) and the next day infected with 4 x 10⁶ cfus that were prepared from a stationary phase culture grown in brain heart infusion at 32 °C without shaking. After a 1.5 hr attachment period excess bacteria were removed and gentamicin was added at 2 µg/ml to kill non-internalized bacteria. After a 1.5 hr extracellular killing period internalized bacteria were enumerated at various time points by lysing the cells with water and determining the colony forming units (cfu) by plating.

**Results**

**The host cellular factor HRI regulates infection-induced TNFα expression**

Prior studies have shown that LPS-induced expression of the proinflammatory cytokine TNFα is reduced in *Hri* ^+/−^ compared to *Hri* ^+/+^ macrophages [13]. To determine whether this defective inflammatory response of *Hri* ^−/−^ macrophages to LPS reflects a role for HRI in the cellular response to infection with bacterial pathogens, TNFα expression was measured in *Hri* ^+/+^ and *Hri* ^−/−^ primary macrophages infected with either *Yersinia pseudotuberculosis* or *Listeria monocytogenes*. In peritoneal macrophages from a *Hri* ^+/+^ mouse infected in vitro with these pathogens, TNFα-encoding transcript levels increased >500-fold following a 3-hr infection period (Figure 1A). In contrast, there was only a modest increase (~3-fold) in the levels of TNFα-encoding transcript in infected *Hri* ^−/−^ peritoneal macrophages. Consistent with the transcript analysis, there was a several-fold increase in TNFα protein levels in *Hri* ^+/+^ splenic macrophages following 5 hrs of infection with these two pathogens whereas there was essentially no changes in TNFα levels in similarly infected *Hri* ^−/−^ macrophages (Figure 1B; data for *Y. pseudotuberculosis* not shown). Previously we showed that an intact eIF2 signaling pathway was required for infection-induced cytokine expression [4]. The findings presented here suggest that HRI is the primary eIF2α kinase responsible for this effect. However, upon closer examination it became apparent that the role HRI plays in the cellular infection cycle of these pathogens far exceeds its regulation of cytokine expression.

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**Figure 1.** HRI is required for infection-induced cytokine expression. (A) Peritoneal macrophages isolated from *Hri* ^+/+^ and ^−/−^ mice were infected *in vitro* with either *Yersinia pseudotuberculosis* (Y.p.) or *Listeria monocytogenes* (L.m.) for 3 hr. Plotted are the levels of TNFα-encoding transcripts in infected *Hri* ^+/+^ and ^−/−^ cells relative to their respective uninfected control cells. (B) Splenocytes isolated from *Hri* ^+/+^ and ^−/−^ mice were either left uninfected or infected *in vitro* with L.m. for 5 hrs and then intracellularly stained for TNFα protein. Histrogram displays TNFa levels in macrophages (live, CD11b^+^-gated). The median fluorescence intensities (MFI) of 3 separate experiments is shown (right panel). *P* values calculated using student *t* test. DOI: 10.1371/journal.pone.0068754.g001

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HRI is required for the function of *Yersinia* T3S system

The pathogenicity of *Yersinia* is inextricably linked to its delivery of virulence factors (or effectors) directly into the host cell cytosol through the T3S system [14]. Upon their delivery into the cytosol, these effectors suppress the induction of host protective cytokine expression via YopJ-dependent mechanisms as well as cause a striking collapse of the cytoskeleton primarily by the actions of YopE [15]. The latter property serves as a readout for T3S activity [16] and can be readily observed in cultured wild-type (Hri +/+ ) mouse embryonic fibroblasts (MEFs) which transition from their normally flatten appearance to a partially detached ‘rounded’ morphology within an hour of infection (Figure 2).

Unsurprisingly, Hri −/− cells displayed almost no overt signs of this cytoskeletal disruption following infection (Figure 2). The resistance of Hri −/− cells to T3S-mediated disruption of the cytoskeleton could not be attributed to differential bacterial adhesion as determined both by direct microscopic examination and plating methods (Figure S1). Furthermore, there were no discernable generalized defects in the cytoskeletal function in the absence of HRI as both Hri +/+ and −/− cells migrated with comparable efficiency in Transwell migration assays (data not shown).

To directly test whether the lack of cytoskeletal disruption in *Yersinia*-infected Hri −/− cells was due to reduced functioning of the T3S system, we used a YopE-Elk translocation assay as a reporter of the delivery of *Yersinia* virulence factors into the host cell cytosol [12]. Compared to Hri +/+ cells, YopE translocated into Hri −/− cells was greatly reduced (Figure 2B; lanes 1 and 2). The YopE translocation defect in Hri −/− MEF cytosol was similar to that observed for a *Yersinia* strain harboring a genetically inactivated T3S system (lanes 3 and 4; ref. [17]). Additionally, this translocation defect in the Hri −/− cells is independent of the Yop virulence factors themselves since this latter assay is performed in a *Yersinia* strain that is genetically deleted for all of the Yop virulence factors. These findings indicate that HRI is required for the T3S-mediated transfer of *Yersinia*-encoded virulence factors into the host cell cytosol. Although host cell processes have been described that promote T3S activity (e.g., the RhoA GTPases [18]), to the best of our knowledge this is the first description of a host factor

![Figure 2.](image-url)
that, when absent, renders a cell refractory to the translocation of T3S effectors.

To date all of the described activity of HRI is associated with it acting as a regulator of protein synthesis by virtue of its eIF2α kinase activity (see Introduction). Previously we reported that protein synthesis rates do not differ between cells infected with wild-type Y. pseudotuberculosis versus a T3S mutant derivative [15] and more recently we showed that there were no differences in Y. pseudotuberculosis T3S activity between infected wild-type host cells and cells expressing an eIF2α with an S51A replacement (the target residue of the eIF2α kinases) [4]. To further test whether T3S activity and protein synthesis in host cells are functionally linked, we treated cells with cycloheximide prior to (and during) their infection with Y. pseudotuberculosis. We observed no differences in either the kinetics or the severity of cytoskeletal disruption between untreated and cycloheximide-treated cells (Figure S2). Collectively these findings indicate that function of the Yersinia T3S system is not coupled to protein synthesis in host cells (or vice versa). Thus, the mechanism by which HRI positively impacts the T3S system in Yersinia infected cells is likely independent of HRI-mediated regulation of protein translation.

**PKR and HRI independently regulate cellular infectivity of Chlamydia trachomatis**

We recently showed that cells expressing an eIF2α with an S51A replacement (the target residue of the eIF2α kinases) were highly colonized by C. trachomatis indicating that this pathway plays a critical role in the complex cellular infection cycle of this bacterial pathogen [4]. Following infection, C. trachomatis forms a membrane-bound inclusion body within which it differentiates from the invasive elementary bodies (EBs) into the proliferating reticulocyte bodies (RBs). To determine whether the eIF2α kinases PKR and/or HRI regulate the infection dynamics of this pathogen, wild-type (+/+ ) and knockout (-/-) cells were infected with C. trachomatis and the number of inclusion bodies (‘direct counts’) were determined. Pkr -/- cells were significantly more permissive for invasion compared to Pkr +/+ cells (Figure 3A). The higher level of invasion of Pkr -/- cells was similar to that we recently reported for cells expressing eIF2α(S51A) [4]. In contrast, there were comparable numbers of inclusion bodies observed in Hri +/+ and -/- cells following infection with C. trachomatis indicating that HRI does not substantially affect Chlamydial infection (Figure 3A). The similar invasion phenotypes of the PKR null and eIF2α(S51A) cells suggest that PKR-mediated activation of eIF2 signaling opposes C. trachomatis invasion.

Although the number of inclusions that formed in Hri +/+ and -/- cells were comparable, there were, however, considerable difference in the sizes of these inclusions. The sizes of the inclusions in the Hri -/- cells were much reduced and these inclusions were populated by much fewer C. trachomatis compared to the Hri +/+ cells (Figure 3B). To quantitatively measure post-invasion proliferation a replating assay is performed in which infected cells are lysed and the yield of infectious units (‘progeny’) is determined. The yield progeny from Hri +/+ cells was ~10-fold greater than that recovered from Hri -/- cells (Figure 3C). Even greater differences (~50-fold) in progeny yields were observed in in vitro-infected peritoneal macrophages isolated from Hri +/+ and -/- mice (Figure 3D). These data show that HRI promotes the post-invasion intra-vacuolar proliferation of C. trachomatis.

Similar to Yersinia, pathogenic Chlamydia employs a T3S system to optimally infect eukaryotic cells [19]. The unaffected invasion rate but reduced intra-vacuolar growth phenotype of C. trachomatis in Hri -/- cells resembles that observed in cells treated with small molecules that disrupt Chlamydial T3SS functioning [20,21]. A high dose infection assay was therefore used to determine whether Chlamydial T3SS activity is reduced in Hri -/- cells. A relatively low multiplicity of infection (MOI) was used in the assays shown above such that cells were likely to be infected with a single EB. In cells infected with >1 EBs, each EB initially forms a single inclusion which eventually fuses with other EB-containing inclusions within the same cell. The fusion of multiple inclusions is abrogated by small molecule inhibitors of the T3S system and is dependent on IncA which is an effector of the C. trachomatis T3S system [22,23]. Cells were infected with a high MOI of C. trachomatis and then examined for the number of inclusions per infected cell. The majority of wild-type infected cells contained a single inclusion whereas Hri -/- cells contained small multiple inclusions consistent with a defect in IncA-mediated fusion activity (Figure S3). Collectively, these data indicate that HRI plays a positive role in the function of the C. trachomatis T3S system.

**HRI is required for efficient trafficking of Listeria to the infected cell cytosol**

The experiments described above indicate that HRI is required for effectors of the T3S system to gain access to the infected cell cytosol. It was next tested whether HRI regulates the cytosolic access of the Gram-positive pathogen Listeria monocytogenes. The intracellular infection cycle of L. monocytogenes consists of three distinct phases: (i) host cell invasion; (ii) vacuole escape of the bacterium to the cytosol; and (iii) intracytosolic association with actin and subsequent proliferation; each of these events is mediated by well-characterized virulence factors [24]. Previously we showed that, like C. trachomatis noted above, L. monocytogenes invaded cells expressing the non-phosphorylatable eIF2α(S51A) at a much higher level compared to wild-type cells [4]. This finding indicated that L. monocytogenes functionally interacts with the eIF2 signaling pathway during the cellular invasion phase of its infection cycle.

An in vitro infection assay was employed to determine whether HRI regulates the intracellular proliferation of L. monocytogenes. Using this assay the initial invasion phase of the infection does not appear to differ between Hri +/+ and -/- cells (Figure 4A; compare the ‘3’ hr time points). Following invasion, L. monocytogenes readily proliferates within Hri +/+ cells increasing ~5-fold after an additional 6 hrs of infection. In contrast, there is a relatively rapid and sustained decrease in the number of viable L. monocytogenes recovered from Hri -/- cells (Figure 4A). This latter infection profile (i.e., normal levels of invasion but defective post-invasion proliferation) is similar to that observed in Hri +/+ cells infected with an attenuated L. monocytogenes mutant strain lacking listerolysin O (LLO)
This mutant strain invades cells but is quickly killed due to its inability to gain access to the cytosol [25]. Additionally, the infection profile of the attenuated LLO mutant strain was similar in Hri +/+ and -/- cells (Figure 4B) indicating that HRI is not required to rapidly eliminate this attenuated strain. These data show that the infection dynamics of virulent L. monocytogenes is greatly impacted by HRI.

It was then tested whether HRI affects the delivery of Listeria-encoded factors to the cytosol. Splenocytes derived from Hri +/+ and -/- mice were infected in vitro with a L. monocytogenes strain expressing ovalbumin (OVA)-peptide. This peptide is a well-defined antigen that is loaded onto class I MHC molecules in the ER and presented on the surface of infected cells. Bacterial-derived OVA-peptide associated with MHC-I could readily be detected on Hri +/+ macrophages following a brief infection. In contrast, the MHC-I associated with OVA was not detected on Hri -/- macrophages (Figure 5A). These results suggest that Listeria-derived factors failed to access host cell cytosol in the absence of HRI.

Next we directly determined whether HRI is required for L. monocytogenes to gain access to the cytosol. Unstimulated peritoneal macrophages derived from Hri +/+ and -/- mice were infected in vitro with L. monocytogenes. There was no appreciable differences between Hri +/+ and -/- macrophages in the levels of internalized L. monocytogenes following brief infection period indicating that, like for the experiment shown in Figure 4 using fibroblastic cells, the invasion phase of L. monocytogenes infection is not regulated by HRI. However, differences between Hri +/+ and -/- cells started to become evident upon prolonged infection periods. Actin-associated L. monocytogenes (which serves as a marker for cytosolic bacteria) are first observed in Hri +/+ macrophages after 2 hr of infection in contrast to Hri -/- macrophages in which L. monocytogenes is exclusively found in vacuoles. After 6 hr of infection there was a significantly higher fraction of actin-associated L. monocytogenes in Hri +/+ macrophages compared to Hri -/- macrophages (Figure 5B). However, a substantial fraction of the bacteria that did make it to the cytosol in the Hri -/- macrophages were competent to form actin tails (Figure 5B) indicating that this post-escape phase of the infection cycle of L. monocytogenes is not regulated by HRI. These observations indicate that L. monocytogenes requires HRI to efficiently traffic to the cytosol.

To determine how HRI impacts Listeria-host cell interactions over longer infection periods, Hri +/+ and -/- cells were infected for 18 hrs and then stained for intracellular vacuoles and actin. There was a much higher level of general cytotoxicity in the infected Hri +/+ cells as evidenced by a greater number of...
detached and rounded cells. Among the surviving \( \text{Hri}^{+/+} \) cells, many contained large numbers of \( L. \text{monocytogenes} \) (Figure S4). Additionally, the vast majority of \( L. \text{monocytogenes} \) in \( \text{Hri}^{-/-} \) cells were within the cytosol. By all measures, \( \text{Hri}^{-/-} \) cells were much less impacted by long-term infection with \( L. \text{monocytogenes} \). There was very little evidence of cytotoxicity in that infected cells maintained their original morphology and a substantial fraction of \( L. \text{monocytogenes} \) did not co-localized with actin (Figure S4, right panels). Collectively, three lines of evidence (bacterial proliferation, antigen processing, and direct observations) indicate that HRI specifically regulates the second phase of the \( L. \text{monocytogenes} \) cellular infection cycle: post-invasion trafficking to the cytosol.

**Discussion**

Here we demonstrate that HRI positively affects the cell-level infection dynamics of three dissimilar bacterial pathogens. The extracellular pathogen \( Yersinia \), the vacuole-bound pathogen \( Chlamydia \), and the cytosolic pathogen \( Listeria \), all require HRI to efficiently complete their respective cellular infection cycles. A common denominator among these three pathogens is that their respective infection cycles are dependent on forming pores in infected host cellular membranes. In Gram-negative pathogens such as \( Yersinia \) and \( Chlamydia \), the T3S secrete translocators that assemble pore-forming structures in the host plasma membrane that mediate the transfer of effectors into the cytosol [14,17]. In some respects this process resembles that which occurs in \( Listeria \)-infected cells. Following its invasion of the host cell, \( Listeria \) secretes monomeric LLO that, following its activation by the host-encoded gamma-interferon-inducible lysosomal thiol reductase (GILT) [26], binds to and oligomerizes into pore-forming structures within the endosomal membrane. In addition to allowing leakage of antimicrobial factors, the resulting pores are also thought to allow the access of co-expressed and secreted phospholipases to the inner leaflet of the endosomal membrane [24,25]. It is possible that one or more of these events occur with reduced efficiency in HRI null cells.

We believe that the activities of HRI described here neither involve it acting as an eIF2\( \alpha \) kinase nor otherwise affecting protein synthesis. That T3S secretion is not coupled to host cell protein translation was indicated by our finding that cycloheximide treatment did not affect YopE-mediated disruption of the host cell cytoskeleton. The most compelling data, in our opinion, supporting the model that the infection-specific activities of HRI are independent of its role as a regulator of protein synthesis is the fact that cells lacking the Ser51 residue of eIF2\( \alpha \) (the phosphorylation site of HRI and the other eIF2\( \alpha \) kinases) are just as competent as wild-type cells in supporting both the T3S activity of \( Yersinia \) as well as the T3S-dependent intracellular proliferation of \( Chlamydia \) [4]. However, eIF2\( \alpha \)(S51A)-expressing cells are more permissive for bacterial invasion indicating that eIF2 signaling does impact the initial...
events of the pathogen–host cell interaction. For example, *Chlamydia* is much more efficient at forming inclusions in eIF2α(S51A) cells; however, the number of infectious EBs per inclusion (a measure of intra-vacuolar growth) is comparable between wild-type and eIF2 α(S51A) cells [4]. This latter phenotype resembles that observed for PKR null cells (Figure 3) indicating that PKR-mediated eIF2 signaling acts to oppose bacterial invasion but does not affect the subsequent maturation of the Chlamydial inclusion. Our findings are consistent with the observation first noted by Alexander [27] of increased Chlamydial proliferation in cycloheximide-treated cells due to enhanced pathogen invasion.

Owing to its broad activity in promoting the intracellular proliferation of pathogens, HRI may be an excellent target for the development of anti-microbial compounds. HRI is an especially attractive target since it is not required for responses to non-pathogens but interferes with specific virulence mechanisms. Recently, it has been shown that HRI activity can be reduced by either direct targeting with small molecules or indirectly by using natural products that inhibit the HRI-cofactor Hsp90 [28–30]. The targeting of host factors may make it less likely that a pathogen would evolve drug-resistance since the pathogen would not have genetic control over the interaction between the compound and its target.

**Supporting Information**

**Figure S1. Comparable adhesion of *Y. pseudotuberculosis* to Hri +/- and -/- cells.** The indicated MEFs were infected...
with *Y. pseudotuberculosis* at a MOI of 5 for 45 min. Cells were then washed several times, lysed, and the resulting lysates were plated on LB media. Two days later the resulting colonies were enumerated and plotted is the average number of colonies from three independently-infected wells. (TIFF)

**Figure S2.** Cycloheximide does not affect the type 3 secretion system of *Y. pseudotuberculosis*. HeLa cells were treated were either treated or not with cycloheximide (25 μg/ml) one hour prior to the addition of *Y. pseudotuberculosis* as well as for 2 additional hours of infection at which time live cells were imagined. (TIF)

**Figure S3.** Delayed fusogency of *C. trachomatis* in *Hri -/-* cells. MEFs were infected at an MOI of 5-10 and visualized 20 hrs post infection. On average one inclusion per cell was detected in *Hri +/+* while multiple inclusions per cell routinely occurred in *Hri -/-* cells. (TIF)

**Figure S4.** *Hri -/-* cells are highly resistant to long-term *Listeria* infection. *Hri +/+* and -/- MEFs were infected with GFP-expressing Lm for 18 hrs and then stained for actin (red), nuclei (purple), and vacuoles (pink). Shown in the enlarged images are numerous actin-associated Lm in *Hri +/+* cells and in the *Hri -/-* cells either non-actin associated Lm (green) or Lm that are lightly associated with actin (yellow). (TIFF)

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**Author Contributions**

Conceived and designed the experiments: NS JB RR KAF WNK KS. Performed the experiments: NS JB WB ESC RR KAF WNK KS. Analyzed the data: NS JB RR KAF WNK KS. Wrote the manuscript: KS.

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