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Chlamydia effector proteins and new insights into chlamydial cellular microbiology

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Chlamydia and *Chlamydophila* sp. are highly related obligate intracellular bacterial pathogens that cause sexually transmitted diseases, ocular infections and atypical pneumonias. Relatively little is known about the molecular mechanisms by which *Chlamydiae* manipulate the mammalian host because they are intractable to genetic manipulation. Studies with heterologous expression systems have revealed a large set of chlamydial proteins that are potentially translocated into the host cytoplasm ('effector' proteins). As new cell biological observations are made and the function of effector proteins begin to be elucidated, a clearer picture of the extent to which *Chlamydiae* manipulate mammalian cellular processes is beginning to emerge, including the cell cycle, innate immunity, and lipid and membrane transport.

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Introduction

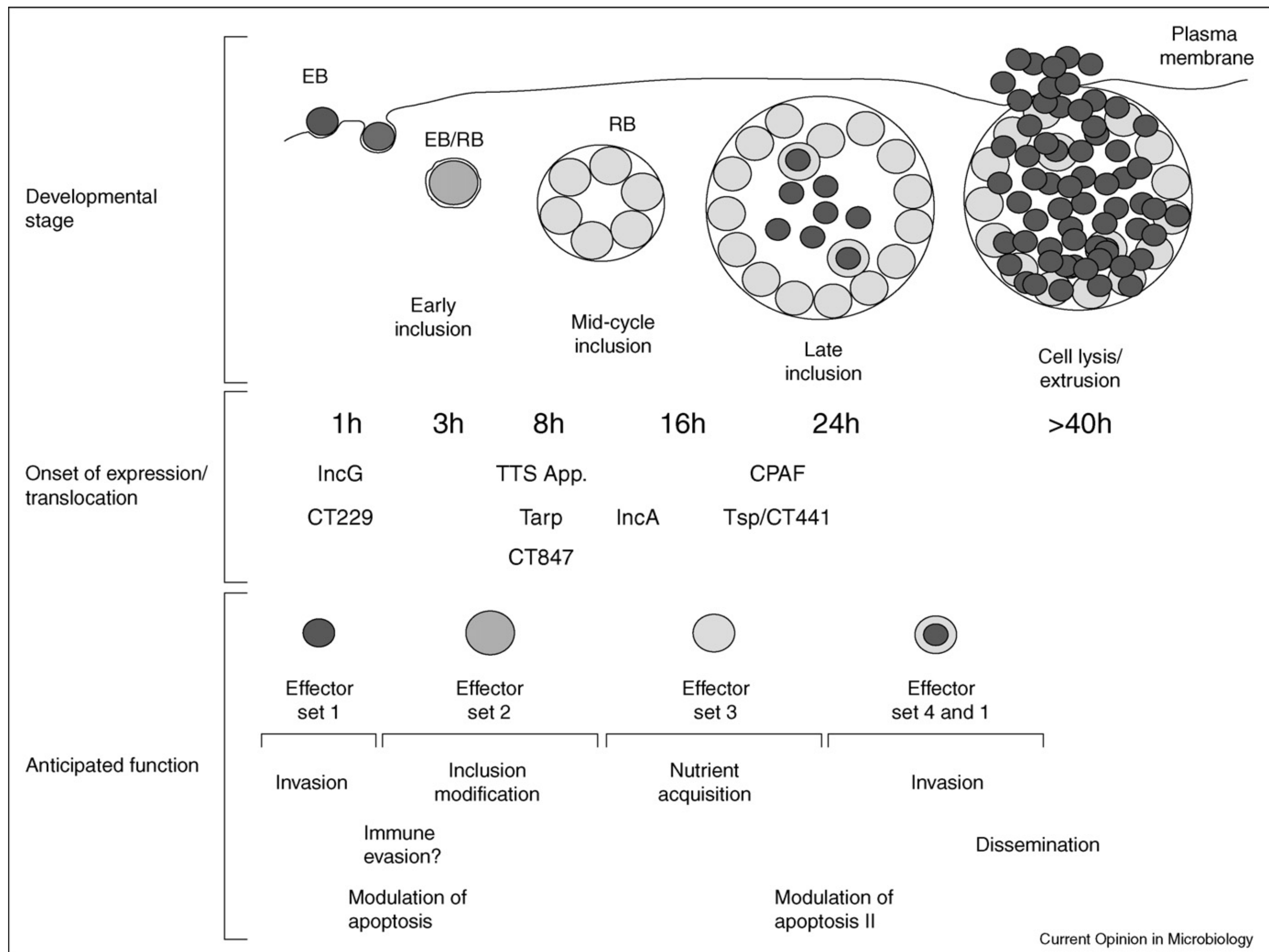
Chlamydia and *Chlamydophila* species are widely disseminated Gram-negative, obligate intracellular bacterial pathogens. In humans, *Chlamydia trachomatis* infects the epithelium of the conjuctiva and the genital tract. Chronic inflammation from recurring *C. trachomatis* infections can lead to severe complications ranging from blindness to pelvic inflammatory disease and infertility [1]. A closely related species, *Chlamydophila pneumoniae*, causes atypical pneumonias and has been linked epidemiologically to atherosclerosis and increased risk of heart disease [2].

Like other Gram-negative pathogens, *Chlamydiae* translocate 'effector' proteins into their host to modulate cellular functions. Unfortunately, the identity and function of many of these effector proteins has remained elusive because *Chlamydiae* are notoriously intractable to genetic analysis. In this review, I highlight progress made in identifying chlamydial effector proteins and recent cell biological findings that have significantly expanded our understanding of how *Chlamydiae* interact with their hosts.

The increasing complexity of *Chlamydia*–host interactions

Chlamydiae have a very complex infectious cycle. Infection begins with the attachment of an elementary body (EB), a metabolically inactive 'spore-like' form of the bacteria, to the surface of epithelial cells (Figure 1). After attachment, *C. trachomatis* induces the localized activation of the Rho-GTPase Rac1, resulting in filamentous actin reorganization and internalization of EBs [3]. At least one chlamydial effector protein, Tarp, is translocated during the entry step to nucleate actin filament formation and promote bacterial entry [4]. Shortly after entry, EBs differentiate into the metabolically active reticulate bodies (RB). The RB-containing vacuole is segregated from normal endosomal maturation pathways to generate a membrane-bound parasitophorous vacuole termed an 'inclusion'. Initially, the *Chlamydia*-containing endosome contains markers of the plasma membrane, however, these markers are shed from the nascent inclusion within 30 min after entry [5]. The inclusion intimately associates with recycling endosomes and recruits the minus-end-directed motor Dynein to migrate along microtubules to the Microtubule Organizing Center (MTOC) [5,6]. During this process, a subset of Rab GTPases, central regulators of membrane transport and organelle identity are recruited to inclusion membranes [7]. Rabs and their associated proteins are likely responsible for imparting the inclusion with its unique ability to selectively interact with host organelles. Interestingly, the association of Rab proteins with inclusion membranes occurs in a species-specific manner with Rab1, 4 and 11 associating with inclusions from all chlamydial species, and Rab6 and Rab10 associating with *C. trachomatis* and *C. pneumoniae*, respectively [7]. These results indicate that there are distinct differences in how chlamydial species interact with intracellular membranes. In fact, it should be noted that because there are significant biovar-specific and species-specific differences in the way *Chlamydiae* interact with host cells, caution should be exercised when extrapolating findings made in one chlamydial species.

Figure 1



The *C. trachomatis* infectious cycle and a model for effector protein function. Infection begins with the attachment of Elementary Bodies (EB) to the surface of epithelial cells. The biogenesis of the nascent inclusion is accompanied by the developmental transition from EBs to Reticulate Bodies (RB) and by the activation of early genes (1–8 h). Mid-cycle genes (8–16 h) accompany the expansion of the inclusion and acquisition of nutrients to support robust replication of RBs. Late in the cycle (16–24 h), RBs replicate asynchronously to generate both RBs and EBs. At this stage, effector proteins required for infection of a new cell are pre-loaded onto EBs and effector proteins required for exit from the mammalian host are assembled. Effector proteins are synthesized and translocated into the host in a temporal fashion to coordinate the stage-specific modulation of cellular functions.

As the inclusion expands, chlamydial replication becomes asynchronous to yield both RBs and EBs and the replicating bacteria acquire energy and biosynthetic precursors from the infected cell. In particular, *Chlamydiae* are adept at acquiring host-derived lipids, including sterols [8], sphingolipids [9], glycerophospholipids [10], and neutral lipids [11••] by vesicle-dependent and vesicle-independent mechanisms [8,12]. In pioneering studies, the Hackstadt laboratory demonstrated that the inclusion intercepted vesicles derived from the Golgi apparatus to acquire sphingolipids and cholesterol [8,9]. However, it was not clear whether fusion of Golgi-derived exocytic vesicles with inclusion membranes could deliver nonlipid biosynthetic precursors to RBs because the protein cargo normally associated with these vesicles is not found in the inclusion [9]. A recent report detailing an interaction between the inclusion and the multivesicular bodies (MVB) pathway, a branch of the late endo-lysosomal system required for the degradation of integral membrane proteins, provides an alternative model for lipid and nutrient delivery to the inclusion [13••]. In this study, the tetraspanin protein CD63, the sterol carrier MLN64 and lysobisphospatidic acid, all markers of MVB, were shown to accumulate in the lumen of *C. trachomatis* serovar E inclusions [13••]. Consistent with a role for MVBs in nutrient acquisition, pharmacological inhibition of MVB formation restricted chlamydial replication [13••]. However, the relative contribution

of these pathways to chlamydial lipid and nutrient acquisition is unclear because some of the inhibitors used to block MVB formation also prevent autophagy [14]. Recent genome wide RNAi screens for host factors required for chlamydial replication did not reveal a role for either autophagy or MVB formation in *C. caviae* replication [15••], suggesting either species-specific effects or redundancy in the mechanisms of nutrient transport. *Legionella pneumophila*, another intracellular pathogen, intersects two branches of ER to Golgi membrane transport and disruption of one branch alone is not sufficient to prevent biogenesis of the *Legionella* replicative vacuole [16]. It would not be surprising if *Chlamydia* had built a similar redundancy into its lipid acquisition options.

Eventually, most of the cytoplasmic space of the host cell is occupied by the inclusion and EBs exit the host cell. The mechanism of chlamydial exit from infected cells is complex with at least two pathways described, cell lysis by the activation of cysteine proteases and by extrusion of the inclusion into the extracellular media by an actin-dependent and myosin-dependent mechanism [17••]. Recently, EB egress in cells infected with *C. trachomatis* serovar E has been shown to be accompanied by lysosome-mediated repair of the plasma membrane [18].

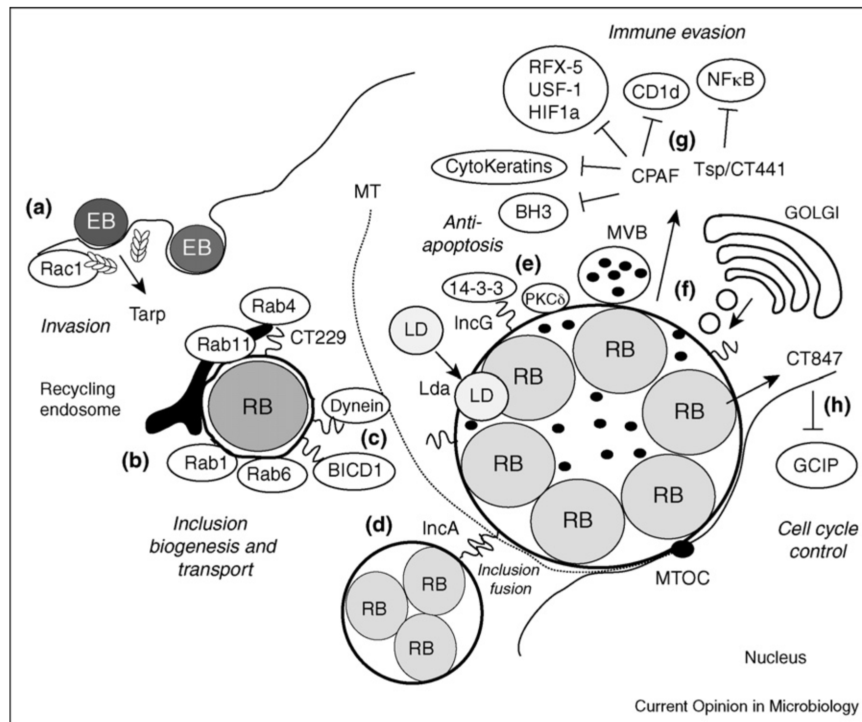
Throughout the infectious cycle *Chlamydiae* modulate many other cellular functions. Prominent among these is the disruption of apoptotic programs that are central to innate immune responses. For example, early in infection, *C. trachomatis* prevents pro-apoptotic phosphorylated BAD and atypical PKCδ from binding to mitochondria by sequestering their binding partners, 14-3-3 proteins and diacylglycerols, respectively [19,20]. At least one chlamydial protein, CADD, can induce cell death when ectopically expressed in mammalian cells by interacting with death domains of TNF family of receptors [21], but the significance of this association is unknown. Later in infection, the secreted chlamydial protease CPAF degrades BH3-only domain proapoptotic proteins [22], ensuring a complete shut down of the infected cell's ability to undergo apoptosis in response to intrinsic and extrinsic stimuli [23]. CPAF further disables adaptive immune responses by degrading factors required for MHC expression (RFX-5 and USF-1) and lipid antigen presentation (CD1d) [24,25]. Finally, *Chlamydia* infections significantly impact the cell cycle of infected cells, with evidence for cleavage of the mitotic cyclin B1 [26], delays in cytokinesis [27] and centrosome supernumeracy [28]. Interestingly, all these later functions can lead to genomic instability, which in conjuction with the strong anti-apoptotic effect of chlamydial infection may explain the epidemiological association between *C. trachomatis* infections and cervical cancers [29].

The role of chlamydial effector proteins in inclusion biogenesis and the modulation of host cellular functions

Studies with inhibitors of bacterial protein synthesis suggest that the modulation of the host cellular function described above requires the activity of chlamydial proteins. All *Chlamydiae* code for the core components of a Type III Secretion (TTS) apparatus [30], a protein transport system used by Gram-negative bacteria to translocate proteins into the cytoplasm of the host cell. Therefore, it is commonly accepted that many chlamydial effector proteins will be targets of TTS. It should be noted that chlamydial effector proteins can also access the cytoplasm of infected cells via TTS-independent mechanisms. For example, CPAF has a Type II secretion signal and is secreted to the inclusion lumen before translocation into the cytoplasm of the infected cell [31].

The first set of chlamydial effector proteins identified was a family of integral inclusion membrane (Inc) proteins that share a large 40–60 aa bi-lobal hydrophobic motif. This motif is a strong predictor of protein localization to the inclusion membrane and suggests that a significant proportion (~5%) of the chlamydial genome codes for proteins that potentially reside at the interface of the inclusion and the host cytoplasm [32]. As such, Incs are probably central regulators of bacterial–host interactions. Indeed, Scidmore and colleagues reasoned that Inc proteins may participate in the recruitment of Rab proteins to the inclusion and identified the Inc CT229 as a Rab4-GTP interacting protein both *in vitro* and *in vivo* [33]. Similarly the Inc, Cpn0585, interacts with Rab1, Rab10, and Rab11 and may mediate their recruitment to the C. *pneumoniae* inclusion [34•]. In some instances, Rab interacting proteins appear to be directly recruited to the inclusion membrane. For example, Bicaudal D1 (BICD1), a Rab6 interacting partner, is recruited to the inclusion independently of Rab6, suggesting that an Inc protein may interact directly with BICD1 [35•]. Other Inc proteins participate in inclusion biogenesis and in modulation of host cellular functions. IncA, for example, mediates homotypic fusion of inclusions [36] potentially by forming a SNARE-like fusogenic intermediate between adjacent inclusions [37], and IncG sequesters 14-3-3β and its proapoptotic-binding partner phospho-BAD [20]. A schematic representation of *C. trachomatis* interactions with host cells is shown in Figure 2. One of the future challenges in deciphering the molecular basis of chlamydial co-option of host cellular functions will be to reconcile why the bacterial proteins identified as responsible for conserved features of chlamydial infections (e.g. Rab recruitment, inhibition of apoptosis) are often not conserved among the *Chlamydiae* (e.g. CT229, IncG). One possibility is that there is a built-in redundancy among Inc genes and that 'subfamilies' of divergent Inc proteins perform overlapping functions. In this manner, the pathogen can be buffered from deleterious

Figure 2



The intersection of *C. trachomatis* and host cell biology. **(a)** Upon EB binding to epithelial surfaces, Tarp is translocated to attachment sites, which in concert with the GTPase Rac1 lead to localized actin rearrangement and internalization of bacteria. **(b)** After entry, the nascent inclusion sheds plasma membrane markers and dissociates from classical endosomal maturation pathways but intimately associates with recycling endosomes and acquires Rab1, Rab4, Rab6 and Rab11 [7]. Rab4 interacts directly with the early Inc protein CT229 [33]. In addition, the Rab6 binding partner BICD1, is recruited independently of Rab6 [35]. **(c)** The microtubule-based motor Dynein associates with the inclusion to direct the transport of inclusion to the microtubule organizing center (MTOC) [6]. Because dynamitin, part of the dynactin cargo adaptor complex, is not required for dynein recruitment to the inclusion, it is predicted that an Inc protein substitutes for this function [6]. **(d)** Inc proteins also mediate inclusion fusion (IncA) and **(e)** the recruitment of 14-3-3 signaling proteins (IncG) important in chlamydial anti-apoptotic functions [20,36]. The sequestration of the pro-apoptotic PKCδ at the inclusion membrane has also been proposed to protect *C. trachomatis*-infected cells from apoptosis [19]. **(f)** The inclusion interacts with various components of the endomembrane system, including Golgi-derived exocytic vesicles, multivesicular bodies (MVBs), and Lipid Droplets (LDs) [8,11,13••]. The interaction between LDs and the inclusion may be mediated by a family of chlamydial LD-associated proteins (Lda) [11]. **(g)** The secreted CPAF and Tsp/CT441 disable innate immune responses by blocking NF-κβ signaling and degrading factors important in immunity [24,44,48]. In addition, CPAF targets pro-apoptotic proteins and modifies cytoskeletal structures [22,49]. **(h)** Finally, CT847 participates in the modulation of the cell cycle by binding to and potentially promoting the degradation of GCIP [43••].

mutations and the development of resistance from the host.

During infection, a gene expression program consisting of early-genes (1–8 h), mid-genes (8–16 h) and late genes (16–24 h) [38] coordinates the transition between chlamydial developmental forms and the synthesis of virulence factors. We envision a scenario wherein the synthesis and translocation of waves of effector proteins is coordinated with the chlamydial infectious cycle and/or in response to cues from the host cell (Figure 1) because the modulation of host cellular functions appears to be orchestrated in a temporal fashion. This hypothesis predicts the following series of events: effector proteins synthesized late in infection are prepackaged into EBs and translocated into the host upon attachment to the epithelial surface. These effector proteins initiate EB invasion, disarm innate immune responses, and delay the maturation of the EB-containing endosome. A second wave of effector proteins, which includes several Inc proteins, is expressed early after invasion and participates in the biogenesis of the nascent inclusion and promotes inclusion migration to the MTOC [6]. Mid-cycle effector proteins are devoted to nutrient and lipid acquisition, manipulation of the cell cycle, and signaling events (e.g. activation of ERK) important in inflammation [39]. Finally, effector protein synthesized late in the cycle prepare the inclusion for exit from the host and to pack EBs with effector proteins required for infection of a new host cell.

Identification of effector proteins using heterologous expression systems

Several studies have used the relative promiscuity of TTS to identify chlamydial proteins that can be secreted by the *Yersinia*, *Shigella*, and *Salmonella* TTS systems

[40••,41,42]. Although these screens have not been performed at a genomic scale, preliminary findings suggest that between 5 and 8% of the chlamydial genome, including several Inc genes, could encode targets of TTS [40••,43••]. This number is probably an underestimate, because many TTS targets may be missed because of specific folding and secretion chaperone requirements or their secretion signals are too divergent for recognition by enteric TTS. Similarly, it is not known how many additional Type II-secreted proteins, like CPAF and Tsp/CT441 [44•], can access the host cytoplasm.

An alternative method to identify effector proteins is to screen chlamydial proteins for discernible biochemical activities. The yeast *Saccharomyces cerevisiae* has emerged as a convenient, genetically tractable model in which to test the function of bacterial virulence proteins [45] because endomembrane, cytoskeletal, and signaling functions are relatively conserved in eukaryotic cells. In a recent study, chlamydial ORFs of unknown function were systematically expressed in yeast and the resulting strains were screened for phenotypes consistent with the disruption of basic cellular functions [46•]. This study identified 34 potential chlamydial effectors, including the TTS substrates Tarp and CopB, based on their ability to inhibit growth and by their tropism for eukaryotic organelles [46•]. Linking the timing of protein expression with TTS screens and any phenotypic information will be useful in defining the role of these effector proteins during infection (Figure 1). For example, proteins transcribed late in infection (>16 hours), which are translocated by TTS and which display a phenotype when ectopically expressed in eukaryotic cells would be excellent candidates as effectors responsible for invasion or nascent inclusion biogenesis. Because of the lack of genetic tools, the translocation of putative effector proteins identified in heterologous expression systems, needs to be confirmed with specific antibodies. In the absence of a known mammalian binding partner, these reagents remain one of the only tools available to determine the relative contribution of translocated effectors in chlamydial infection [36].

New insights into *Chlamydia* cellular microbiology from effector protein function

As new effector proteins are identified and hints to their function determined, it is apparent that there are aspects of chlamydial biology that have been previously overlooked. For example, the identification of *Chla*Dub1–2, *C. trachomatis* proteins with de-ubiquitinating activity [47], suggests that *Chlamydia* may regulate ubiquitin-dependent protein degradation, signaling, and vesicular transport. Similarly, the identification of Grap cyclin D interacting protein (GCIP) as a binding partner of the conserved TTS effector protein CT847, and the observation that GCIP is degraded during infection suggests that *Chlamydiae* manipulate the proliferative capacity of their host cells [43••].

An analysis of chlamydial proteins ectopically expressed in eukaryotic cells revealed a subset of *C. trachomatis* proteins with tropism for Lipid Droplets (LDs), a neutral lipid storage organelle, and prompted further studies on the role of neutral lipids in chlamydial infections [11••]. *C. trachomatis* infection disrupted neutral lipid homeostasis and pharmacological inhibition of neutral lipid biosynthesis negatively impacted chlamydial replication [11••]. Strikingly, electron and live cell microscopy revealed that cytoplasmic LDs cross the inclusion membrane and intimately associate with RBs (unpublished observations). The molecular basis for this unusual example of organelle subversion remains to be determined. Chlamydial LD-associated (Lda) proteins, which localize to the cytoplasmic face of the inclusion membrane [11••] are likely to participate in the capture and translocation of these organelles into the inclusion lumen.

Even effector proteins such as CPAF, whose role in infections was thought to be well understood, have revealed new surprises. Recent findings linking CPAF to the cleavage of the hypoxia-induced transcription factor, HIF1α, in *C. pneumoniae*-infected cells [48] and intermediate filaments [49], a central component of the mammalian cytoskeleton, suggest that CPAFs role in infection extends beyond modulating innate and adaptive immune responses. Indeed, as new targets of CPAF are identified, our models of *Chlamydiae* interactions with host cells will need to be updated.

Conclusions

Identifying the function of effector proteins has been pivotal to our understanding of bacterial pathogenesis. The experimental toolkits available to identify and characterize chlamydial effector proteins has significantly expanded with the advent of comparative genomics, DNA microarrays, and genome-scale protein expression systems. Despite the lack of tools for genetic manipulation, an integration of diverse approaches, including new cell biological tools and functional genomics in the pathogen and the host, will lead to significant breakthroughs in our understanding of chlamydial biology. Given that *Chlamydiae* arrived at a genetic solution to successful intracellular parasitism early in eukaryotic evolution [50], we predict that these pathogens will reveal novel and unique aspects of cellular microbiology.

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