

Virulence News & Views

Is nectin-1 the “master” receptor for deadly herpes B virus infection?

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The family Herpesviridae is a large, diverse family of double-stranded enveloped DNA viruses. Herpes B virus (BV) and Cercopithecine herpesvirus 2 (CeHV-2) are simian herpesviruses. Like herpes simplex virus 1 (HSV-1), they belong to the α -herpesvirus subfamily. Interest in BV infections results from the observation that zoonotic infections often result in death of humans, whereas infection of macaques, the natural host of BV, results in disease similar to that observed on HSV infections of humans. We recently reported in cell-cell fusion assays in which nectin-1, a HSV-1 gD receptor, mediated fusion of cells expressing glycoproteins from both BV and CeHV-2. However, HVEM, another HSV-1 gD receptor, did not mediate fusion by BV and CeHV-2 glycoproteins. Paired immunoglobulin-like type 2 receptor α (PILR α), an HSV-1 gB fusion receptor, did not mediate fusion with BV or CeHV-2 glycoproteins. These results were further confirmed by BV infection. Our results may indicate that differential receptor usage by BV in humans when compared with macaques may have pathological consequences. Understanding human and simian receptor usage for BV and HSV may provide clues to understand the pathogenesis of these viruses, as well as related viruses, in their natural host as well zoonotic infections. This broader understanding may result in the development of novel therapeutics to control deadly BV infections as well as herpesvirus infections in general.

Herpes B virus (BV, officially named as Macacine herpesvirus 1, formerly Herpesvirus simiae, monkey B virus, or Cercopithecine herpesvirus 1) and Cercopithecine herpesvirus 2 (CeHV-2, formerly simian agent 8) are primate herpesviruses belonging to the α -herpesvirus subfamily and are closely related to herpes simplex virus 1 (HSV-1) and HSV-2. HSV causes recurrent mucocutaneous lesions on the mouth, face, or genitalia and in rare cases can cause meningitis or encephalitis. BV naturally infects macaques whereas zoonotic infections of foreign hosts, such as humans, can result

in encephalitis, encephalomyelitis and death. BV infection has high mortality in humans (greater than 50% in documented infections) and as such is recognized as a deadly virus for humans requiring biosafety level 4. Similarly to the high level of pathogenicity of BV in humans, HSV infection of marmosets can also be fatal. CeHV-2 is a pathogen of baboons and is not known to cause disease in primates outside the natural hosts. Since the receptors for HSV are well described, we chose to explore the receptor usage of the BV and CeHV-2.

HSV entry into target cells requires a minimum four virus encoded glycoproteins—glycoproteins B (gB), D (gD), H (gH) and L (gL). These glycoproteins interact with a variety of cellular receptors to facilitate virus entry. Best described are the cellular receptors for gB and gD which we chose to study. The interaction of gD with the gD receptors has been extensively investigated. The binding of gD to herpesvirus entry mediator (HVEM) (Montgomery et al., *Cell* 1996), nectin-1 (Geraghty et al., *Science* 1998), nectin-2 (Warner et al., *Virology* 1998) and modified heparan sulfate (Shukla et al., *Cell* 1999) triggers fusion of the virion envelope with a cellular membrane. Paired immunoglobulin-like type 2 receptor α (PILR α) is a receptor that binds to gB and also mediates entry and fusion (Satoh et al., *Cell* 2008) even though it shows much less fusion activity than the gD receptors nectin-1 and HVEM. Two other gB receptors, with less known in regard to the significance in virus infection, are myelin-associated glycoprotein (MAG) (Suenaga et al., *Proc Natl Acad Sci U S A* 2010), and non-muscle myosin heavy chain IIA (NMHC-IIA) (Arii et al., *Nature* 2010). HVEM is a member of the tumor necrosis factor receptor family. Nectin-1 and nectin-2 are cell adhesion molecules in the immunoglobulin superfamily and are widely expressed by a variety of cell types, including epithelial cells and neurons. Specific sites in heparan sulfate generated by certain 3-O-sulfotransferases (3-O-S HS) can

also serve as a gD-binding entry receptor. PILR α is expressed on cells of the immune system. MAG is a cell-surface molecule belonging to immunoglobulin superfamily, is usually expressed in neural tissues. NMHC-IIA not only functions in the cytoplasm but also functions on the cell surface with gB upon viral entry. NMHC-IIA is expressed in a broad range of cell lines, tissues and cell types in vivo.

To begin our studies, we generated expression constructs for the homologous glycoproteins (gB, gD, gH and gL) necessary for HSV-1 fusion from BV and CeHV-2. We then performed cell-cell fusion assays to determine which cellular and viral proteins were required for cell-cell fusion. The results indicated that nectin-1 is the primary receptor that mediates fusion for BV and CeHV-2, and that HVEM and PILR α do not function for BV and CeHV-2. To verify our cell-cell fusion results, we used cells expressing human nectin-1, HVEM or PILR α and determined if they could be infected with BV. The results confirmed that BV only utilizes nectin-1 and not HVEM or PILR α . To confirm that BV and CeHV-2 do not utilize human HVEM as an entry receptor because of differences between human and simian HVEM, we performed cell-cell fusion assays with simian HVEM. In these experiments, we found that simian HVEM could mediate fusion with cells expressing HSV-1 glycoproteins however; simian HVEM did not mediate fusion of cells expressing glycoproteins from BV and CeHV-2. Thus, as found in earlier studies and reports from other investigators, our findings are compatible with the idea that nectin-1 is a pan α -herpesvirus entry receptor.

Of particular interest from our recent studies is the idea that differential receptor usage may be important for pathogenesis in infected hosts. Compatible with this idea, studies in mice have shown that nectin-1 is the primary receptor responsible for the infection of the vaginal epithelium with HSV-2. Interestingly, in experimental vaginal infections, nectin-1 was not the sole receptor capable of enabling

spread of HSV infection from the vaginal epithelium to the PNS and CNS (Taylor et al., Cell Host and Microbe 2007). However, expression of nectin-1 is necessary for HSV-2 infection via the intracranial route and for encephalitis while HVEM was not important (Kopp et al., Proc Natl Acad Sci U S A 2009). In contrast, in HSV-1 infection of the murine eye, we found that both HVEM and nectin-1 must be present for maximal HSV-1 infection further suggesting that receptor requirements for HSV depends on the route of infection and/or serotype (Karaba et al., J Virol 2011). The host immune response may also be important in

pathogenesis. Previous studies have shown that engagement of HVEM by gD alters the immune response following murine vaginal infection. In these studies, there was a transient increase in mucosal chemokine and IL-6 levels when compared with infection with wild-type control virus in agreement with gD-HVEM interaction elicits an innate response (Yoon et al., PLoS One 2011). Overall, our studies, as well as studies by others, indicate that choice of receptor utilized as well as the host may be important determinants in regard to the outcome of the infection. Virus receptor usage, replication and control by the host

immune are likely fine-tuned to allow maximal virus replication without being detrimental to the host to allow virus spread to naive hosts. In contrast, when a virus infects a non-natural host, this balance may not exist resulting in severe pathogenesis as observed in BV infections of humans. By understanding the required interactions between host receptors and the relevant glycoproteins required for infection, novel therapeutics might be developed to treat zoonotic infections and prevent deadly infections or more broadly used in natural HSV infections that routinely occur within the human population.

Architecturally the same, but playing a different game: The diverse species-specific roles of DivIVA proteins

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Homologs of the *Bacillus subtilis* DivIVA protein can be found in a wide variety of Gram-positive bacteria. DivIVA proteins are coiled-coil proteins that bind to the cytosolic face of the cytoplasmic membrane and accumulate at membrane regions with higher curvature. By directly interacting with downstream proteins they serve as spatial regulators of other cellular processes. Initial DivIVA studies focused on its role as a topological determinant for the MinCDJ division inhibiting complex in *B. subtilis*, but recent evidence suggests that DivIVA can fulfill more diverse roles in different species of the firmicutes and actinomycetes and can even be relevant for virulence.

DivIVA proteins are highly conserved among different Gram-positive bacteria. They are lipid binding proteins sensitive to membrane curvature and accumulate at areas of the cytoplasmic membrane that are more strongly bent (concave) (Lenarcic et al., EMBO J 2009; Ramamurthi and Losick, Proc Natl Acad Sci U S A 2009). Zones with higher membrane curvature occur at the cell poles of non-cocoid bacteria and establish along the invaginating membrane ring at the site of cell division. DivIVA orthologs of different bacteria were shown to localize to these subcellular regions even when expressed heterologously in other taxa suggesting that membrane binding and curvature sensitivity is an intrinsic feature of DivIVA and does not require the

assistance of other factors (Edwards et al., EMBO J 2000). As an autonomously localizing protein, DivIVA acts as a topological determinant for different cellular processes. DivIVA of *Bacillus subtilis* functions as a spatial regulator of the FtsZ-inhibiting MinCD proteins and restricts their activity to the polar and septal regions of the cell, preventing division in the chromosome-free spaces near the poles and in the vicinity of an existing Z-ring (Marston et al., Genes Dev 1998; Fig. 1A). During sporulation of *B. subtilis*, however, polar localized DivIVA tethers the origin region of the spore chromosome to the distal pole of the prespore. This polar origin recruitment is vital for the correct transfer of one chromosome copy from the mother cell into the developing spore compartment and is thus essential for sporulation (Thomaidis et al., Genes Dev 2001; Fig. 1A). Both functions involve direct protein-protein interactions of DivIVA to specific binding partners: The trans-membrane protein MinJ is the molecular bridge mediating the contact between DivIVA and MinD to ensure polar and septal recruitment of MinCD (Bramkamp et al., Mol Microbiol 2008; Patrick and Kearns, Mol Microbiol 2008), whereas RacA acts as a sporulation specific DivIVA binding partner (Ben-Yehuda et al., Science 2003; Wu and Errington, Mol Microbiol 2003). This protein binds to 14 bp inverted repeats scattered around the chromosomal origin and also binds directly

to DivIVA (Ben-Yehuda et al., Mol Cell 2005; Lenarcic et al., EMBO J 2009).

In a recent study (Halbedel et al., Mol Microbiol 2012) we have analyzed the function of DivIVA in the facultative human pathogen *Listeria monocytogenes*, which is closely related to *B. subtilis*. To our surprise, deletion of the *divIVA* gene in *L. monocytogenes* caused a phenotype that was different from the classical filamentous and mini-cell producing phenotype of a *B. subtilis* Δ DivIVA mutant. In contrast, Δ divIVA mutants of *L. monocytogenes* grew as long chains of cells that had clearly completed cell division, which however remained attached even after completion of cross wall synthesis. Electron microscopy revealed that the daughter cell cytoplasm of the Δ divIVA mutant were precisely separated at the division site and a cross wall was made in-between them. But whereas wild-type cells degrade this cross wall to physically separate the daughter cells, cross walls of the Δ divIVA mutant showed no signs of degradation. Obviously, deletion of *divIVA* had affected the post-divisional separation of daughter cells and not cell division per se. Since *L. monocytogenes* encodes two autolysins, p60 and MurA, that play a role in cross wall turnover (Pilgrim et al., Infect Immun 2003; Carroll et al., J Bacteriol 2003), we tested their production in the Δ divIVA background. We found that secretion of p60 and MurA was severely impaired in Δ divIVA cells and

this result led us to the accessory secretion ATPase SecA2. Accessory SecA2 ATPases are encoded by a number of Gram-positive pathogens in addition to the canonical housekeeping SecA and specifically help to translocate a small subset of secretory proteins across the cytoplasmic membrane. Both listerial autolysins are known substrates of this secretion route (Lenz et al., Proc Natl Acad Sci U S A 2003) and therefore it appeared plausible that DivIVA somehow is involved in this. We tested this concept by comparing the secretion defect of the $\Delta secA2$ deletion strain with the $\Delta divIVA$ mutant. And indeed, both mutants were similarly deficient in p60 and MurA secretion and accumulated the precursors of p60 and MurA inside their cytoplasm to the same extent. A microscopic analysis of the subcellular localization of p60, MurA and SecA2 gave us the first hint as to how DivIVA might act at the molecular level. GFP-tagged versions of p60, MurA and SecA2 localized to the division sites, suggesting that SecA2-dependent secretion of p60 and MurA occurs at the septum. In a $\Delta divIVA$ mutant however, p60-GFP and MurA-GFP are diffuse whereas the subcellular distribution of SecA2 was unaffected. One possible interpretation is that DivIVA might recruit the pre-proteins of p60 and MurA to the invaginating membrane ring that occurs at the division site as soon as the divisome starts to constrict (Fig. 1B). Septal autolysin secretion might be one mechanism to restrict the activity of p60 and MurA to the freshly synthesized cross wall peptidoglycan that is the substrate of both autolysins. Cells lacking DivIVA fail to target the secretion substrates to the translocon, hence secretion of autolysins cannot occur. This role of DivIVA in SecA2-dependent autolysin secretion had pleiotropic implications for the biology of *L. monocytogenes*: likewise to naturally occurring *secA2* mutants or deletion mutants in *secA2* (Lenz and Portnoy, Mol Microbiol 2002), $\Delta divIVA$ cells formed rough colonies. They cannot grow as a biofilm and were non-motile even though flagella were clearly assembled on the cell surface. In vitro

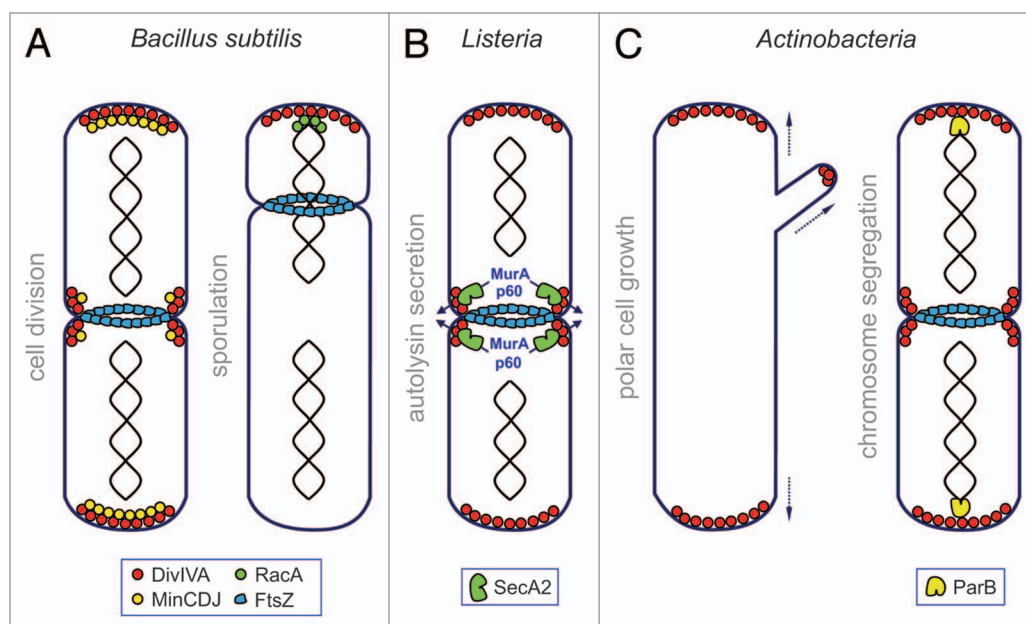


Figure 1. Models illustrating the diverse DivIVA functions in different Gram-positive bacteria. (A) *B. subtilis* DivIVA recruits the Z-ring inhibitors MinCDJ away from mid-cell in order to ensure Z-ring formation in the chromosome free space at mid-cell. During sporulation the prespore chromosome is tethered to the prespore pole via RacA which binds to polar DivIVA. (B) *L. monocytogenes* DivIVA is required for efficient protein secretion via the accessory secretion ATPase SecA2. DivIVA recruits the autolysins p60 and MurA to the division septum from where they are secreted in a SecA2-dependent manner. Please note that septal localization of SecA2 is independent of DivIVA. (C) In different actinobacteria, DivIVA marks the site of cell wall synthesis at the poles and the hyphal tips indicating that DivIVA recruits cell wall biosynthetic proteins to these subcellular regions. DivIVA of several actinobacteria furthermore interacts with ParB, an origin binding protein, in order to recruit the origin to the cell poles. It is believed that this event is required for efficient chromosome segregation.

infection models revealed that $\Delta divIVA$ cells were severely attenuated in terms of invasion and cell-to-cell spread and the severity of these effects was similar to those seen with a $\Delta secA2$ mutant strain. Most likely, the formation of long cell chains impairs the ability of *L. monocytogenes* to enter eukaryotic host cells and prevents coordinated intracellular motility and thus bacterial spreading in host tissues.

These data already indicated that the function of DivIVA proteins might be quite diverse even in closely related species. However, another recent publication (Donovan et al., Mol Microbiol 2012) further illustrates the diversity of DivIVA functions. In the actinomycete *Corynebacterium glutamicum*, DivIVA is supposed to act as a polar origin tethering factor during chromosome segregation (Fig. 1C). This was deduced from experiments where DivIVA and the centromere-binding protein ParB were expressed as fluorescently tagged versions in *Escherichia coli*. Polar and septal localization of ParB-CFP clearly required the presence of DivIVA in the heterologous *E. coli* background. ParB binds to 16 bp direct

repeats, called *parS* sites, near the origin of the *C. glutamicum* chromosome, and was found to accumulate close to the cell poles in *C. glutamicum* cells (Donovan et al., J Bacteriol 2010). The *divIVA* gene could not be deleted from the *C. glutamicum* genome suggesting that it codes for an essential cellular function (Ramos et al., Microbiology 2003) but depletion of DivIVA resulted in coccoid cells, which normally would grow as rods (Letek et al., J Bacteriol 2008). Whether polar localization of ParB is lost and chromosomes are less efficiently segregated under this condition has so far not been addressed experimentally. But expression of a mutant ParB protein that no longer binds to DivIVA (ParBR21A) in cells of *C. glutamicum* resulted in a clear chromosome segregation defect as many anucleate cells were produced. In line with these results, ParBR21A fused to CFP was less frequently recruited to the cell poles (Donovan et al., Mol Microbiol 2012). Taken together these data support a model in which polar localized DivIVA is used to attach the chromosomal origins to the cell pole via the centromere-binding protein ParB and this

event facilitates chromosome segregation in *C. glutamicum* (Fig. 1C). The same study provided evidence for similar interactions between the DivIVA homolog of *Mycobacterium tuberculosis* Wag31 and its cognate ParB protein, as well as between DivIVA and ParB from *Streptomyces coelicolor* (Donovan et al., Mol Microbiol 2012). Hence, the role of DivIVA in polar recruitment of ParB likely represents a conserved feature of the actinobacteria.

Morphological aberrations upon DivIVA depletion are also seen in other actinobacteria in which the *divIVA* gene is essential such as *S. coelicolor* and *Mycobacterium smegmatis*. The reason for this is that cell growth in actinobacteria is brought about by polar cell wall elongation, which is mediated by the DivIVA-dependent recruitment of the peptidoglycan biosynthetic apparatus to the poles of the cell (Flårdh, Mol Microbiol 2003; Kang et al., Microbiology 2008; Letek et al., J Bacteriol 2008; Hempel et al., J Bacteriol 2008). It is presently unknown which exact peptidoglycan biosynthetic proteins are recruited to the cell poles by actinobacterial DivIVA proteins. However, two emerging candidates are the cellulose synthase like protein CslA of *S. coelicolor* and the penicillin-binding protein PBP3 of *M. smegmatis*. CslA mainly accumulates at the hyphal tips of the *S. coelicolor* mycelium and is involved in the deposition of β -1,4-linked polysaccharides at the tips of the hyphae. CslA interacts with DivIVA in a bacterial two-hybrid assay (Xu et al., J Bacteriol 2008) but evidence that polar recruitment of CslA requires DivIVA is still lacking. Such analyses are hampered in actinobacteria by the severe morphological deformations in DivIVA depleted cells which

make it hard to conclude whether a possible protein localization defect is due to the lack of DivIVA or caused by alterations of cell shape. The interaction between PBP3 and the DivIVA-homolog Wag31 from mycobacteria was shown by a multitude of approaches including co-immunoprecipitation, bacterial two-hybrid analysis, and surface plasmon resonance experiments (Mukherjee et al., Mol Microbiol 2009). Likewise to the situation in *S. coelicolor*, both PBP3 and Wag31 can be found at the cell poles (Datta et al., Mol Microbiol 2006; Nguyen et al., J Bacteriol 2007) but the interdependence of their subcellular localization patterns still has to be addressed experimentally. Interestingly, Wag31 needs to be in the periplasm to interact with PBP3 since mutations in the extracellular transpeptidase domain of PBP3 abolished the Wag31-PBP3 interaction. Wag31 was indeed detected in the extracytosolic fraction of *M. smegmatis* (Mukherjee et al., Mol Microbiol 2009). These findings, however, challenge our current view on DivIVA proteins that are supposed to be non-secreted cytosolic factors. The exciting question as to how a cytosolic protein that lacks a signal sequence can be transported across the membrane, still awaits further clarification.

What the DivIVA proteins from all the mentioned species have in common is their unique localization to curved membrane regions at the cell poles and the division site, as well as their conserved domain arrangement. What distinguishes them, however, is the physiological context they work in and the proteins they bind to. Their ability to attach to membranes is mediated by a highly conserved N-terminal lipid binding domain (LBD) that is believed to

interact with membranes via the insertion of surface exposed hydrophobic amino acid side chains into the likewise hydrophobic core of the phospholipid bilayer (Lenarcic et al., EMBO J 2009; Oliva et al., EMBO J 2010). Via a flexible linker of varying length, the LBD is connected to an α -helical C-terminal domain (CTD) that is rich in coiled coils (Oliva et al., EMBO J 2010). Monomers of *B. subtilis* DivIVA assemble into a parallel dimer by means of a coiled coil in the LBD and due to the coiled coil regions in the CTD. Based on analytical ultracentrifugation it was concluded that DivIVA forms tetramers in solution (Oliva et al., EMBO J 2010). Consistently, crystallographic data revealed that formation of the DivIVA tetramer arises from dimerization of two DivIVA dimers that are arranged in an end-to-end orientation. It is tempting to speculate that this tetramer represents the basic building block of higher order DivIVA aggregates since assembly into string- and network-like ultrastructures was indeed observed in cryo-negative stain transmission electron microscopy experiments with a mutant variant (E162K) of *B. subtilis* DivIVA (Stahlberg et al., Mol Microbiol 2004). But this mutant protein was described as a 6- to 8-mer in solution and thus behaved different from wild-type DivIVA. All in all, however, DivIVA homologs are two-domain proteins that consist of an invariant N-terminal membrane-binding module fused to variable, species-specific CTDs. It therefore appears plausible that these regions are important for the interaction of DivIVA homologs with their species-specific binding partners and it will be interesting to determine the structural basis of this diversity.

Long range transcriptional control of virulence critical genes in *Mycobacterium tuberculosis* by nucleoid-associated proteins?

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Identifying novel transcription factors required for virulence may lead to the identification of new drug targets. We have found that transcription of a virulence-critical operon of *Mycobacterium tuberculosis* is regulated by a novel transcription factor binding far upstream of the promoter. This operon includes the *espA*

gene, which is required for ESX-1 secretion. This system has to be precisely regulated since continued secretion of its highly antigenic substrates would alert the immune system to the infection. Transcription of this operon is positively regulated by the EspR transcription factor, and dependent upon far-upstream

sequences containing known EspR binding sites. Transcriptional activation therefore takes place over long distances of the chromosome, by looping out of DNA between the EspR binding site and the promoter. EspR thus has DNA binding characteristics similar to those of nucleoid-associated proteins (NAPs) and joins

two other NAP-like proteins, Lsr2 and CRP^{Mt}, in regulating expression of this operon.

Although virulence in *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is multifactorial, requiring the participation of several cell wall and cellular components, the ESX-1 secretion system is one component that is essential for a successful infection. In fact, the genes coding for parts of this secretion system, in the RD1 region, are deleted in the attenuated vaccine strain of *M. bovis*, BCG, and restoring the RD1 region to BCG results in partial reversal of attenuation. This secretion system, also known as a type VII system, transports certain proteins, notably ESAT-6 and CFP-10 (also known as EsxA and EsxB) into host cells, which appears to affect the phagosome membrane, to facilitate dissemination of *M. tuberculosis* into the cytoplasm. There is also another distinct genetic locus, the *espACD-Rv3613c-Rv3612c* operon, which is required for ESX-1 function (and thus *espA* mutants are attenuated in a mouse infection), and which is expressed in a transient manner during infection of the host cell, the macrophage. An unusual and notable feature of ESX-1 is the mutually dependent nature of the secretion, such that the secretion of each substrate relies on the secretion of the other substrates. Because some of the members of the *espACD-Rv3613c-Rv3612c* operon are themselves substrates, then ESX-1 secretion could be controlled by the short-lived expression of this operon that occurs during infection. Because the ESX-1 secreted proteins are antigenic to the adaptive immune system it is therefore advantageous to the bacterium to switch off the system immediately after phagocytosis by the macrophage.

The *espACD-Rv3613c-Rv3612c* operon is thus vital to ESX-1 secretion, and hence to the virulence of *M. tuberculosis*, and clearly precise control of its expression is required for a successful infection. The chromosomal context of this operon is notable in that the upstream region is relatively large, there being 1,357 bp from the start of the *espA* open-reading frame (ORF) to the start of the divergently transcribed *Rv3617* ORF, suggesting that there is enough DNA to accommodate numerous regulatory elements. In fact several DNA binding proteins have been shown to regulate expression of this operon. Thus it has previously been found that the mycobacterial cyclic AMP receptor protein (CRP^{Mt}) (Rickman et al., Mol Microbiol 2005) and the nucleoid-associated protein Lsr2

repress transcription of this operon (Gordon et al., Proc Natl Acad Sci U S A 2010), and the PhoP (Walters et al., Mol Microbiol 2006; Frigui et al., PLoS Pathog 2008) and EspR (Raghavan et al., Nature 2008) proteins have been found to be likely activators. We first became aware that sequences far upstream of the first gene in the operon, *espA*, were affecting transcription when we made transcriptional promoter fusions. By making a series of such constructs with progressively longer fragments of DNA upstream of *espA*, we were surprised to notice that there were stepped increases in promoter activity as measured by assays for β -galactosidase, the product of the *lacZ* gene, when longer fragments of upstream DNA were fused to the reporter gene. Thus, the first 800 bp upstream of *espA* supported relatively low expression, but when this was increased to include the 800–995 bp region there was a six-fold enhancement in expression. We designated this latter region as the *espA* Activating Region (EAR). More detailed analysis showed that the critical region lay between 887 and 995 bp, with an especially large increase associated with the 11 bp between 984 and 995 bp. Curiously, and perhaps significantly, the presence of DNA from 1,100–1,200 bp reduced activity to an intermediate level.

We knew that these increases in activity were not due to new promoter activity since we showed that the only promoter in the 1,357 bp intergenic region was located 67 bp upstream of the *espA* gene. So the EAR looked much like an enhancer, an invertible, mobile DNA sequence that increases gene expression by mediating RNA polymerase assembly at a promoter. Promoter activity could be decreased by deletions within the EAR (between 993 and 1,003 bp), but it could also be decreased by inserting foreign DNA downstream of the EAR, suggesting that the EAR had been moved too far from the *espA* promoter at –67 bp. Addition of the EAR to a 520 bp fragment of the upstream region resulted in a sizeable increase in activity, but not if it was added to a 217 bp fragment. Moving the EAR construct further away in the 217 + EAR construct by adding a 661 bp foreign DNA fragment did not increase promoter activity, showing that the failure of the EAR to act from 217 bp was not because it was too close and unable to loop out. So we concluded that the 217 bp fragment lacked a further site required for the EAR, although it is possible that the insert disrupted a genetic element in the

217 bp fragment. Nevertheless, the EAR was not a classical enhancer since inverting it abolished its enhancing effect on transcription.

Our conclusion was that the enhancing effect of the EAR could involve the binding of a protein to this site, but which one? As mentioned previously, one of the DNA binding proteins previously found to be an activator was EspR. To test the likelihood that EspR was binding to the EAR we constructed an *espR* deletion mutant of *M. tuberculosis* using homologous recombination, and used this as a host for different length *espA* promoter fusions. From this experiment it was evident that the increases in promoter activity that resulted from adding longer fragments of upstream DNA were dependent upon a functional *espR* gene. We concluded that EspR must be mediating the increased transcription that resulted from increased length of the *espA* upstream region, thereby enhancing promoter activity, by binding far upstream of the start of transcription at –67 bp.

Significantly, while we were completing these experiments, another group published the location of EspR binding sites in the *espA* upstream region (Rosenberg et al., Proc Natl Acad Sci U S A 2011). These were reported to be at 468 bp, 798 bp and 983 bp upstream of the *espA* gene; the position of the latter two high affinity sites correlated very closely with the results of our in vivo transcription fusion experiments where there was a particularly large increase in promoter activity when the upstream DNA fragments were extended from 800 bp to 995 bp. These results therefore provided a molecular explanation for our in vivo transcription activity data. The binding site identified was non-palindromic, which would explain why the EAR did not function when inverted.

As mentioned above, it seemed curious that transcription reporter gene activity decreased when longer fragments of upstream DNA were fused to the *lacZ* reporter gene. A possible explanation of this has now appeared from a recent publication by Blasco et al. (PLoS Pathog 2012) who have mapped the EspR binding sites using ChIP-seq experiments. This has shown that there is another major EspR binding site between 1,113 bp and 1,214 bp upstream of *espA*: perhaps EspR binding at this site has an inhibitory role whereas binding at the other sites activates transcription.

Interestingly, the DNA upstream of *espA* containing the EspR-binding sites is missing

from some members of the *M. tuberculosis* complex. For example, in *M. bovis* the length of DNA upstream of *espA* is only 465 bp rather than the 1,357 bp in *M. tuberculosis*. This has previously been noted as the RD8 deletion. It implies that the sequences upstream of -465 bp relative to the start of the *espA* gene, are not required for virulence since *M. bovis* is still a virulent strain. By examining the sequences of many members of the *M. tuberculosis* complex, it appeared most likely that the common ancestor of the members of the *M. tuberculosis* complex had the complete 1,357 bp upstream region and this had been reduced by two independent deletions, one leading to the evolution of certain members of one lineage coming mainly from the Philippines with an upstream sequences of 288 bp, and another resulting in the evolution of strains related to *M. bovis* having a sequence of 465 bp. We found that the core promoter of *M. bovis*, although having three single nucleotide polymorphisms, had a similar activity to the core promoter of *M. tuberculosis*; nevertheless with the longer upstream region containing the EspR binding sites, transcription emanating from the *M. tuberculosis* sequence was approximately three times greater than that from the *M. bovis* sequence. The consequent differences in expression of the ESX-1 secretion system among these members of the *M. tuberculosis* complex may contribute to their differing pathologies.

Studies of the crystal structure of EspR reported in the paper by Rosenberg et al., mentioned above, and by Blasco et al. (Mol Microbiol 2011) have provided intriguing evidence of how this protein is binding to the upstream DNA sites. These studies have shown that EspR contains an N-terminal helix-turn-helix domain and an atypical C-terminal dimerization domain, resulting in a dimer of dimers with two subunits binding two consecutive major grooves. This means that the other two DNA-binding domains can form higher order oligomers, enabling EspR to bridge distant DNA sites in a cooperative manner, promoting looping of DNA, providing a structural explanation for the results of our in vivo gene expression experiments; in fact Blasco et al. were able to observe such looping using atomic force microscopy with EspR-DNA complexes of the 1,357 bp DNA region upstream of *espA*.

All of this evidence demonstrating that EspR could influence gene transcription by

binding at a considerable distance from the promoter indicated that EspR was not a conventional transcription factor interacting with RNA polymerase at a promoter, but suggested it was more similar to a nucleoid-associated protein (NAP). These proteins can bind to DNA at multiple sites resulting in the bending of DNA: they are the functional equivalent of histones in eukaryotes, organizing higher-order structures of chromosomes, although they do not share structural properties with them. Although some, like HU in *Escherichia coli*, bind DNA in a sequence-independent fashion, others such as IHF bind to well conserved DNA sequences; in this respect EspR appears to resemble the latter. NAPs can often also influence transcription via multiple possible mechanisms, including in some cases displacing an activator, or even interacting with RNA polymerase like more conventional transcription factors, in other cases by assisting contact between regulatory proteins and RNA polymerase. In many cases these proteins have the ability to introduce bends in the DNA that facilitate protein-protein interactions in the nucleoprotein complexes.

So how is EspR activating transcription from the *espA* promoter? As previously mentioned, we know that *espA* transcription is influenced by at least three other proteins: it is activated by another transcription factor PhoP, and repressed by CRP^{Mt} and possibly by the known NAP, Lsr2. One plausible model is that the bending of DNA catalyzed by EspR facilitates the contact between a transcription factor, such as PhoP, and RNA polymerase. In the case of CRP^{Mt}, we know that it can act as a conventional transcription factor at some genes in *M. tuberculosis* by interacting with RNA polymerase at sites close to the promoter (Stapleton et al., J Biol Chem 2010), but in the *espA* upstream region CRP^{Mt} binds at a site over 1,000 bp away from the transcription start site (M. Stapleton, C. Kahramanoglou, unpublished data). In *E. coli*, CRP has also been shown to bind to hundreds of sites along the chromosome and may have many of the characteristics of NAPs (Grainger et al., Proc Natl Acad Sci U S A 2005); based on this paradigm, therefore, one possibility is that CRP^{Mt} can also act as an NAP at some loci such as *espA* to influence gene expression at long range, perhaps in this case by displacing EspR.

Also like many other known NAPs, binding of EspR is not restricted to a single site, the *espA* upstream region. Thus Blasco et al.

(PLoS Pathog 2012) showed that EspR binds to at least 165 genetic loci with binding not being restricted to promoter regions. Like other NAPs, the levels of EspR in the cell are about 30-fold higher than those of classic transcriptional activators, well in excess of that necessary to occupy all of the 582 experimentally detected (within the 165 loci) or the 1,026 computationally predicted binding sites (Blasco et al., PLoS Pathog 2012). It appears to act as an activator of transcription of some genes, but as a repressor of others. Where it does bind to promoter regions, many of the genes concerned have cell wall associated functions, including other ESX secretion systems, and some have known functions in virulence determination, such as the *fadD26* gene involved in the synthesis of the wax-like compound phthiocerol dimycocerosate (PDIM). The reduced virulence of *espR* mutants could therefore be the result of multiple defects rather than solely via *espA* and ESX-1 secretion. In this respect it resembles CRP^{Mt} which also occupies numerous binding sites (C. Kahramanoglou, unpublished data) and affects the expression of many genes; similarly the reduced virulence of *crp* mutants could be the result of multiple defects. Besides *espA*, some other genes regulated by CRP^{Mt}, such as *fadD26*, are also regulated by EspR. Moreover, there is a considerable overlap (77%) between the genes in the EspR regulon and those controlled by the known NAP Lsr2, although as Blasco et al. note, the regulatory outcomes are likely to be different since the binding mechanism of these two proteins differ, with Lsr2 binding to the minor groove of DNA and EspR binding to the major groove. Thus it appears that *M. tuberculosis* has evolved mechanisms involving NAPs to coordinately regulate varied sets of genes at distant sites on the chromosome.

EspR has previously been reported to be secreted by the ESX-1 system (Raghavan et al., Nature 2008). This created a negative feedback loop that modulated the activity of this secretion system. However, the more recent study of Blasco et al. (PLoS Pathog 2012) did not substantiate the claim that EspR is a secreted protein. Nevertheless, a feedback mechanism does probably regulate the levels of EspR, but one based on the control of transcription rather than secretion, since Blasco et al. found that EspR binds to its own promoter to downregulate

its expression, and EspR protein levels are growth phase-dependent, reaching a maximum in stationary phase, at which stage *espR* transcription is lower.

A hallmark of *M. tuberculosis* infection is the ability to form latent infections through the formation of granuloma in the lungs. These dormant bacteria, which have a low metabolic activity, tend to be inaccessible to therapy

by most antibiotics and form a reservoir of infection that can be reactivated especially after disturbance of the immune system, such as occurs after HIV infection. Since it is thought that one-third of the world's population is infected with *M. tuberculosis*, mostly latently, the potential for new tuberculosis cases through reactivation of latent infections is huge. An interesting prospect therefore is

that NAPs are involved in the reorganization of chromatin and subsequent critical changes in gene expression that must occur after the reactivation of dormant bacteria. This process probably needs to be very closely controlled and this would therefore make EspR, Lsr2 and CRP^{Mt} truly key players in the pathogenesis of *M. tuberculosis* and, as such, potential drug targets.

Influenza A viruses and PI3K: Are there time, place and manner restrictions?

Comment on: Ayllon J, et al. *J Virol* 2012; 86:5366–70; PMID:22345452;

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Influenza A viruses activate the host phosphoinositide 3-kinase (PI3K) signaling pathway at several stages of their replication cycle, which depending upon the stage can have pro- or anti-viral consequences. For example, transient PI3K activation due to virion attachment appears to promote virus entry, while pathogen recognition receptor-mediated PI3K signaling may contribute to host innate immune defenses. In addition, the viral NS1 protein, a multifunctional virulence factor, directly binds and activates PI3K to enhance efficient virus replication. Here, we comment on the strain-specific viral requirement for NS1-activated PI3K in vitro and in vivo. Our observations with NS1 suggest that to clarify the complex interplay between PI3K signaling and viral activators, it is essential to understand the temporal and spatial patterns of kinase activation, as well as the specific PI3K subclass and enzyme isotypes involved. It is likely that each independent activation event regulates individual pathways with distinct biological outcomes.

Introduction

The last decade has witnessed a tremendous research effort to identify human host cell factors that regulate virus replication and virulence. Motivated by the need to find new antiviral targets for viruses that currently cause public health concerns (e.g., HIV, hepatitis C and influenza), virologists are reporting increasing numbers of virus-host protein:protein or protein:nucleic acid interactions, as well as multitudes of host genes and pathways that are important for determining the outcome of infection. These data have

largely come from large-scale proteomics (e.g., yeast-2-hybrid analyses or affinity purifications combined with mass spectrometry), transcriptomics and genome-wide siRNA screens. The hope is that by finding ways to manipulate these virus-host interactions, novel intervention strategies can be identified. However, the sheer amount of data generated, together with subtle experimental differences, and the intrinsic complexities of cellular signaling networks, create a challenge for virologists when trying to understand the biological relevance of such findings. One example of this in the influenza A virus field is the role of the phosphoinositide 3-kinase (PI3K) pathway, a critical signaling checkpoint that regulates diverse cellular processes, including growth, survival, translation, vesicle trafficking, membrane biology and immunity. Components of PI3K-regulated signaling consistently appear as critical factors required for the influenza A virus replication cycle in genome-wide siRNA screens. Functionally, there is growing evidence to implicate specific PI3K pathways in the endocytic uptake of influenza viruses. Furthermore, several viral products have been proposed to directly or indirectly modulate the levels of PI3K activity during infection, which confusingly appear to have either pro- or anti-viral consequences. In this News and Views article, we briefly review the complexities of the PI3K signaling pathway and the different ways that influenza viruses interact with this important cellular regulator. We focus on our recent study regarding direct activation of PI3K signaling by the influenza A virus NS1 protein, a multifunctional virulence

factor. Our data have led us to hypothesize that for NS1-mediated PI3K signaling to have a pro-viral function, activation must occur at a distinct sub-cellular location and involve a specific PI3K regulatory isoform. We discuss the implications of these findings for our understanding of all PI3K signaling events during influenza A virus infection. We propose that spatial and temporal kinetics intersect with isoform-dependent signaling to delineate separate biological outcomes for each PI3K activation event.

Class IA PI3K: Signaling Inputs and Outputs

Phosphoinositide 3-kinases (PI3K) are a family of cellular enzymes that phosphorylate the 3-hydroxyl group on the inositol ring of membrane-embedded phosphatidylinositol lipids, thereby generating second messenger molecules that regulate the intracellular distribution and activity of various cell signaling proteins. Biological processes regulated in this way broadly include cell growth, survival, proliferation, intracellular trafficking, cytoskeletal rearrangements and migration. Class I PI3K comprises obligate heterodimeric protein complexes consisting of a catalytic subunit (termed p110) and a regulatory subunit (p85-type, p101 or p87), and use phosphatidylinositol (4,5) biphosphate [PtdIns(4,5)P₂] as their main substrate. Subclass IA PI3K includes those catalytic subunit isotypes (p110 α , p110 β and p110 δ) that bind the p85-type (p85, p55 and p50) regulatory subunits. As each of the two main p85-type regulatory subunits (p85 α and p85 β) can heterodimerize with any of the three p110 catalytic subunits, there are at least six types of p85-mediated class IA PI3K

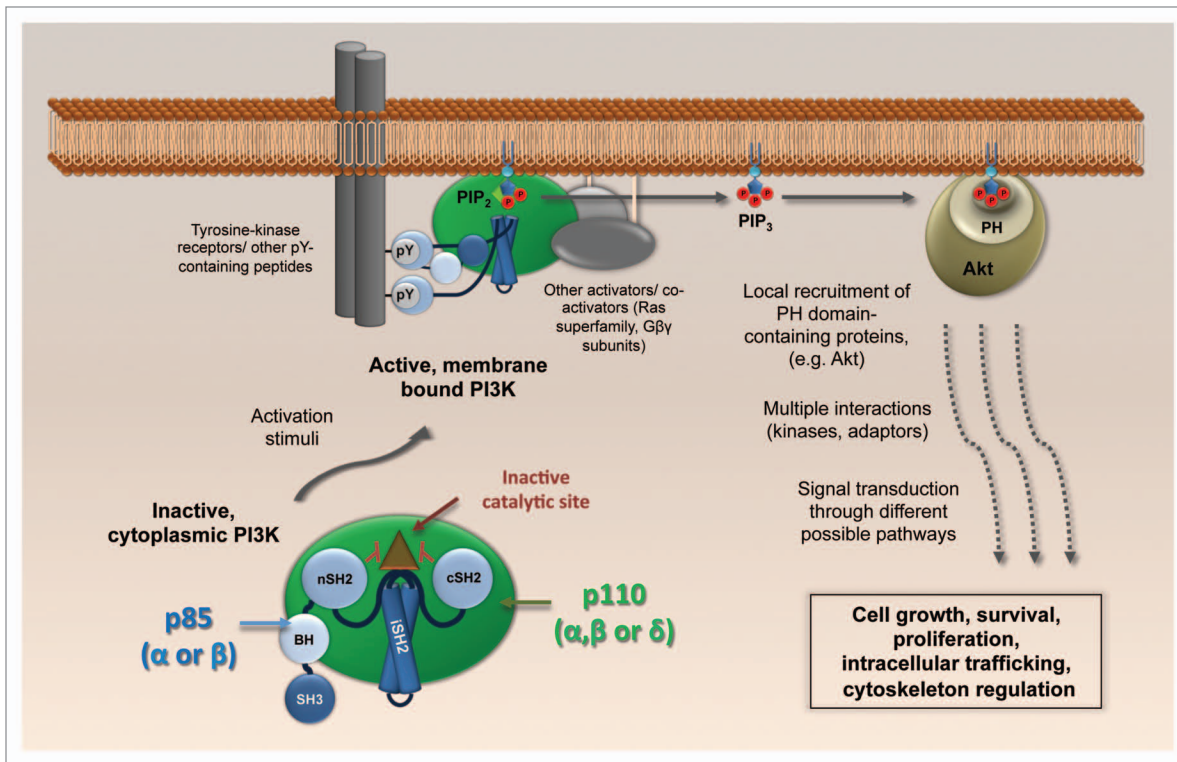


Figure 1. Physiological activation of class IA PI3K. The p110-p85 heterodimers, cytoplasmic in their basal state, dock to cellular membranes upon activation of various signal receptors, including receptor tyrosine kinases. A conformational change in p85 relieves the p110 catalytic site from inhibition by the p85 SH2 domains, and PtdIns(3,4,5)P₃ is generated. Akt (and others with pleckstrin homology (PH) domains) bind to PtdIns(3,4,5)P₃ and build up local signaling platforms for multiple cellular pathways. SH3, Src-homology 3; BH, breakpoint-cluster region homology; nSH2, N-terminal Src-homology 2; iSH2, inter-SH2; cSH2, C-terminal Src-homology 2.

heterodimer. Nevertheless, each heterodimer utilizes the same substrate in the same enzymatic reaction to produce the same second messenger product. Mechanistically, it is unclear how this same reaction output can be translated into biologically distinct outcomes, but given the conservation of different p85 and p110 isoforms and data from genetic ablation studies in mice, it is obvious that functional output differentiation must exist.

Canonically, in the absence of a stimulus, PI3K p110-p85 heterodimers remain inactive in the cytoplasm: the default function of the regulatory p85 subunit is to inhibit the catalytic p110 subunit via specific intermolecular contacts. Upon various stimulatory signaling inputs, including external stimuli such as growth factors or cytokines, the inactive complex moves to the plasma membrane, where the SH2 domains of p85 dock with phosphotyrosine residues present in receptor-associated signal-transduction proteins (summarized in Fig. 1). This leads to a rearrangement of the intermolecular interactions between p85 and p110, such that the enzymatic activity of p110 is no longer inhibited, thereby stimulating

production of the intracellular second messenger, phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P₃], from PtdIns(4,5)P₂. Downstream of PI3K activation, several effector proteins that possess pleckstrin homology (PH) domains [or other PtdIns(3,4,5)P₃-regulated domains] are recruited to the newly generated PtdIns(3,4,5)P₃, and nucleate a plethora of different signaling cascades.

The Ser/Thr kinase Akt is the best-studied PH domain-containing effector downstream of PI3K, and its activating phosphorylation at Ser-473 following PtdIns(3,4,5)P₃ binding has been used as a universal cell-based reporter for PI3K activity. However, several other mechanisms exist to increase PI3K versatility. The variety of PH domain-containing proteins besides Akt greatly expands the range of signals that can be propagated from a single second messenger such as PtdIns(3,4,5)P₃. As stated above, the conservation of different p85 and p110 isoforms must lead to distinct activities, although these are poorly understood. Nevertheless, there is some evidence to suggest a p85-specific bias in directing the PI3K heterodimer toward distinct interaction partners, such

as certain growth factor receptors that have been activated by specific ligands. Moreover, although PtdIns(3,4,5)P₃ is mainly generated at the plasma membrane, it can also be found on nuclear and endocytic membrane compartments. Thus, the intracellular distribution of PtdIns(3,4,5)P₃ pools could influence effector protein composition at particular sites, thereby differentially regulating functional propagation of PI3K signaling. Furthermore, in addition to their lipid kinase activity, several p110 subunits have protein kinase activity that may be specific to certain cellular substrates, and there have been proposals that some p110 subunits have distinct non-catalytic, scaffold-like activities. Direct activation of small GTPases (Ras, Rac and Arf superfamilies) by PI3K has also been described. In summary, the cellular PI3K system is a highly regulated system that can discriminate inputs from a variety of extracellular stimuli and convert them into specific functional outputs. The multitude of PI3K isoforms, their intracellular locations and their differential protein/lipid kinase or non-catalytic activities likely contribute to this.

PI3K Activation during Influenza A Virus Infection

Influenza A viruses are the etiological agents of an acute respiratory disease in humans. These viruses cause both seasonal outbreaks and occasional pandemics, and remain a major global health concern despite our improving knowledge on their biology, and the development of intervention strategies such as vaccination. Influenza A viruses encode 10–12 polypeptides on their 8-segmented, negative-sense RNA genome. Given this small coding capacity, the virus is highly dependent upon, and must interact extensively with, various host-cell signaling components to influence both the viral replication cycle and disease outcome. As described below, the PI3K signaling pathway is an intriguing example of this, as influenza A viruses appear to encode several factors that stimulate activation of this pathway.

Initial studies on the effect of influenza A virus infection on PI3K signaling (by monitoring Akt Ser-473 phosphorylation levels) detected two temporally regulated waves of activation. The first wave appears to be weak, transient, and occurs very early during infection (~15–30 min post-infection). Some studies have proposed that influenza virus particle attachment to sialic acid-containing receptors on the surface of cells it is entering triggers the clustering of receptor tyrosine kinases, thereby mimicking canonical activation of class IA PI3K by growth factors. This, in turn, may promote endocytosis and contribute to acidification of late endosomes by activating v-ATPases, a critical step in triggering virus-host membrane fusion and the release of incoming viral genomes into target cells. Furthermore, Ras-mediated activation of another PI3K class (IB) has also been suggested to potentiate clathrin- and Akt-independent endocytosis of influenza viruses.

The second wave of PI3K activation during influenza A virus infection occurs at intermediate-to-late time points (~4–6 h onwards), is characterized by high levels of phosphorylated Akt and is mediated by expression of the viral NS1 protein, a multifunctional virulence factor with well-defined roles in antagonizing the host innate immune system. NS1 specifically and directly binds the p85 β regulatory subunit of PI3K, but is unable to bind p85 α , an intriguing observation for which the reasons are unknown. In vitro assays have subsequently indicated that such an interaction

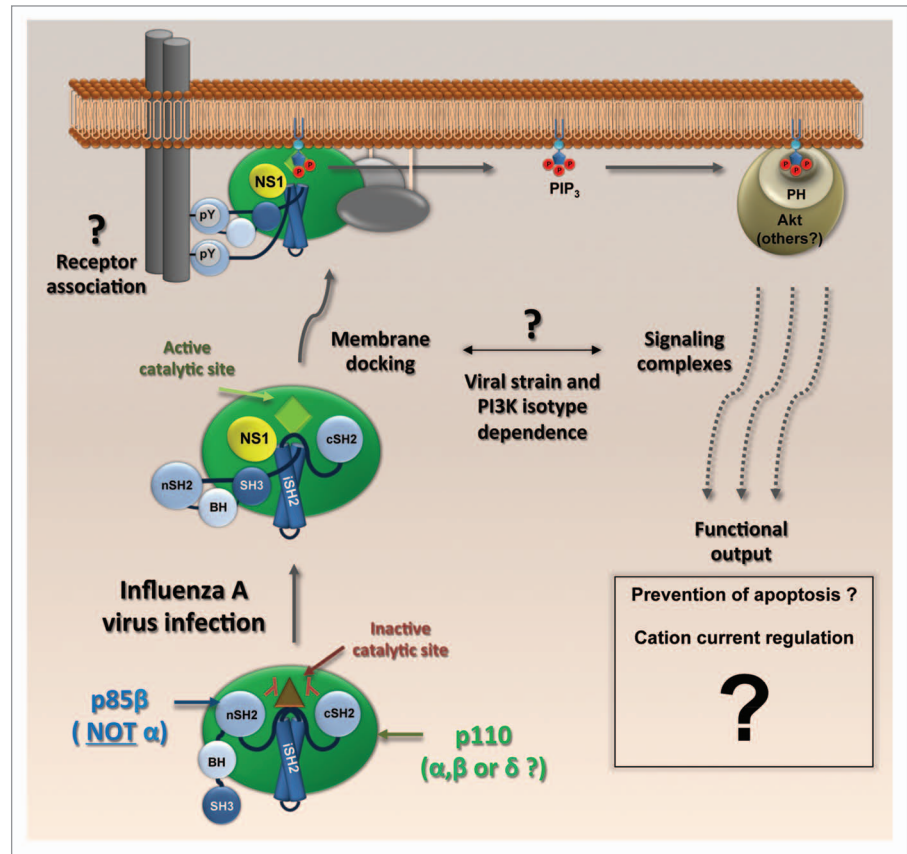


Figure 2. A model of NS1-activated PI3K. NS1 binds the iSH2 domain of p85 β and displaces the inhibitory nSH2 domain. The docking of NS1-activated PI3K complexes to membranes, the involvement of effectors other than Akt and the ultimate biological consequences remain unclear. Different viral strains and PI3K isotypes may drive the response through distinct pathways.

forms the core of a heterotrimeric complex between NS1, p85 β and an inhibition-relieved, catalytically active p110 (Fig. 2). Structural, functional, and biochemical studies have characterized the interacting residues in both NS1 and p85 β . Among them, the highly conserved tyrosine at position 89 of NS1 (Y89) is critically positioned at the interface between NS1 and p85 β . Conservative substitution of tyrosine 89 to phenylalanine (Y89F) abrogates the NS1-p85 β interaction, inhibits PI3K/Akt activation and attenuates virus growth in tissue culture. Perhaps unsurprisingly, given the underlying complexity of the PI3K network and its multiple functional outputs, addressing the biological relevance of PI3K activation by NS1 has been a challenging question. Early work suggested a link between NS1-mediated PI3K activation and a delay in virus-induced apoptosis, which is consistent with a clearly established anti-apoptotic role for the PI3K/Akt pathway. However, other studies have provided data to indicate that even though NS1 has anti-apoptotic properties, these are independent of PI3K

activation, at least in the context of the influenza virus strains tested so far. Another study, more focused on tissue-specific activities, indicates that NS1-activated PI3K can play a role in regulating cation currents (and thereby liquid accumulation) in the respiratory epithelium of the lung. Many factors may account for these discrepancies, not least experimental variation in the viral strains and cell types used, the NS1 mutations that abrogated p85 β binding, and the use of pharmacological inhibitors with unknown specificities and off-target effects.

Although NS1 appears to be the main activator of PI3K during influenza A virus infection, other viral products also stimulate this signaling pathway, thereby adding layers of complexity. In particular, viral genomic RNA (vRNA) may trigger a PI3K-dependent antiviral response leading to production of interferon. Such vRNA-stimulated PI3K signaling seems to be mediated by the host viral RNA sensor, RIG-I, and should have a biological effect (e.g., anti-viral) opposite to that of NS1-activated PI3K (e.g., pro-viral). An unresolved question

is therefore how absolute activation of PI3K by distinct viral inputs can lead to varying consequences for the virus at different stages of the replication cycle.

Strain-Specific Consequences of NS1-Activated PI3K: Not All PI3K Events are Equal?

Previous studies had shown that introduction of the NS1-Y89F mutation into an H3N2 strain of influenza virus (A/Udorn/72) abrogates PI3K activation and attenuates virus replication during infection of tissue culture cells. We were initially interested in assessing the contribution of NS1-activated PI3K to influenza virus virulence in vivo, something that had not been previously assessed. For this purpose, we used the well-established mouse model of influenza A virus infection and introduced the NS1-Y89F mutation into two widely used mouse-lethal H1N1 strains of influenza virus: A/Puerto Rico/8/34 (PR8) and A/WSN/33 (WSN). In tissue culture, the mutant PR8 virus (rPR8 NS1-Y89F) was clearly attenuated as compared with its wild-type counterpart. However, there was no appreciable difference between the wild-type and mutant WSN viruses, despite clear differential activation of PI3K. Given the variety and cell type-specific activities of PI3K, we hypothesized that the effect of NS1-activated PI3K could be limited in the homogeneous context of tissue culture, but still have a significant impact within a complex animal model. Hence, it was surprising to find that the phenotypic difference between strains was maintained in vivo: mice infected with rPR8 NS1-Y89F showed less morbidity and mortality than those infected with wild-type rPR8, while no significant differences were observed between the wild-type and mutant WSN viruses. In accordance with this, mutant PR8 virus grew to lower titers in mouse lungs than wild-type, but there was no significant difference in lung titers between wild-type WSN and the NS1-Y89F mutant.

Both PR8 and WSN NS1 proteins are able to bind p85 β and promote phosphorylation of Akt. Furthermore, it is clear that the Y89F mutation abrogates binding and activation of PI3K for both strains. Thus, it was intriguing to find that removal of the PI3K activating phenotype from these two strains lead to different functional consequences. Interestingly, we found a clear difference between PR8 and WSN in the intracellular localization of PtdIns(3,4,5) P_3 (the catalytic product of activated PI3K) after co-expression of each NS1 protein with p85 β and p110 α . Cells transfected with PR8 NS1

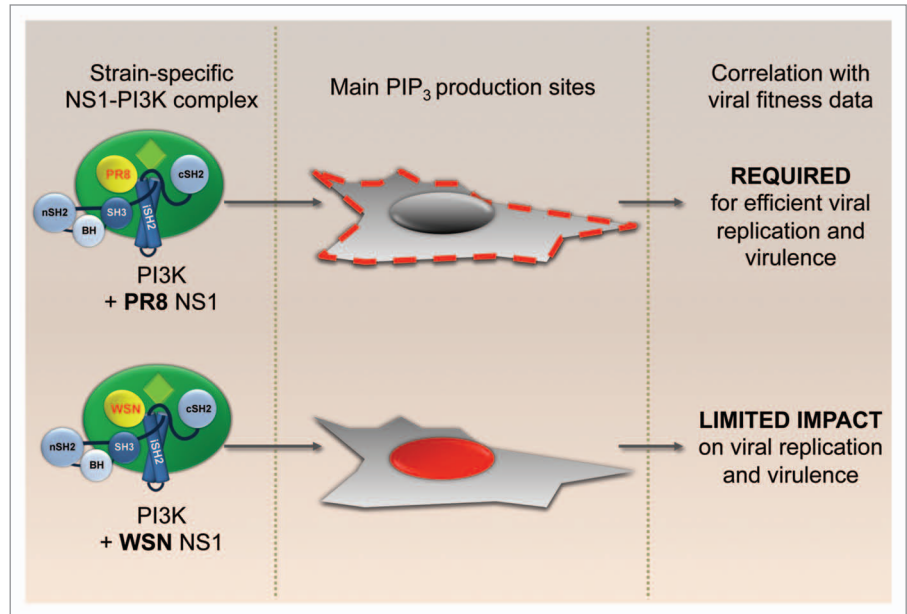


Figure 3. Strain-specific localization of PI3K activation. Viral strain specificity of PtdIns(3,4,5) P_3 generation patterns by NS1-activated p110 α -p85 β complexes and their correlation with virus performance profiles in vitro and in vivo.

showed increased levels of PtdIns(3,4,5) P_3 accumulating in discrete domains of the plasma membrane that we have yet to identify, while WSN NS1-induced PtdIns(3,4,5) P_3 preferentially accumulated in nuclear and perinuclear areas and was not detectably enriched at the plasma membrane. From these results, we postulated that the subcellular location of PI3K activation defines the functional output. In other words, although the initial players involved in the process remain the same [i.e., NS1, PI3K, PtdIns(3,4,5) P_3 and Akt], the pathways they activate downstream (together with their respective biological consequences) depend on the spatial platform from which they are signaling. Thus, we hypothesize that the PI3K signaling cascade initiated at discrete domains of the plasma membrane has a different function to that initiated in the perinuclear/nuclear regions. Accordingly, the PR8 strain is sensitive to defects in activating this specific pathway, while the WSN strain is not (Fig. 3). Presumably, the WSN strain does not require plasma membrane-mediated NS1-activated PI3K for its replication strategy, and it is possible that the ability of this NS1 protein to activate PI3K is a remnant of viral requirement prior to laboratory and mouse adaptation. Multiple issues remain to be elucidated, including the molecular mechanism behind the strain-specific NS1 behavior (our data suggest that NS1 carries the information

required to drive PI3K activation to a particular location), the identity of different platform-specific pathways activated and the reason for the apparent lack of biological relevance for the WSN NS1-induced perinuclear/nuclear accumulation of PtdIns(3,4,5) P_3 . At this point we cannot rule out that WSN NS1-activated PI3K may have biological consequences for the virus in other experimental systems beyond tissue culture cell-lines and murine models.

Widening the Scope beyond Location, Location, Location?

We believe that our observations on the strain-specific spatial activation of PI3K by NS1 and the assumed different signaling and biological consequences this has on virus replication are applicable to our understanding of other PI3K signaling events during influenza virus infection. Thus, future studies on PI3K activation should address spatial distribution during infection and focus on specific subcellular events rather than broad global reporters such as total Akt phosphorylation. In this way, we may begin to resolve the functional consequences of PI3K activity downstream of such diverse stimuli as virion binding, NS1 and vRNA-mediated activation of RIG-I. It may be that the other activation events also have strain-specific outcomes that need to be considered, and it will certainly be important to identify the kinetics of activation with each stimulus and combine this with the spatial

data. For example, given that NS1 location varies over the course of infection, different NS1-PI3K signaling foci could be activated successively by the NS1 protein of a single viral strain, with each focus leading to a different biological outcome.

Another critical parameter that has yet to be taken into account regarding influenza A virus-mediated PI3K activation is the actual variety that exists within the generic term "PI3K." An increasingly important area of research in the PI3K field is therefore to understand the different roles of various PI3K isoforms. In this regard, the functional consequences of NS1 specificity toward activating PI3K complexes that contain p85 β , but not p85 α , are not understood. Whether there is preferential, parallel, redundant or synergistic activation of the different catalytic p110

subunits by NS1 is also a pertinent question, especially given the tissue and cell specificity of these isoforms and the array of different cell types that influenza A viruses can infect in a host organism. This gap needs to be addressed not only for NS1, but also for the other viral activators of PI3K. Due to their clinical relevance in cancer and chronic inflammation, much effort has been put into developing better PI3K (usually p110) isoform-specific inhibitors and antibodies, many of which are now commercially available. Their use may help to clarify specific signaling activities and functional phenotypes associated with the diverse p110-p85 complexes.

Concluding Remarks

To fully understand how influenza A viruses interact with the host PI3K network, multiple variables must be considered. These include

viral strain, host-cell type, time post-infection, subcellular location of the activation events and the PI3K isoforms involved. The consequences of successfully portraying a clear picture of this virus-host interaction could reach far beyond the virology field, given the relevance of PI3K for almost all physiological and pathological processes from development to oncogenesis. As viruses have been "learning" the intracellular molecular biology of our cells for a lot longer than academic biologists, the nuances and specificities of viral proteins such as the influenza A virus NS1 protein may become invaluable tools with which to probe cell signaling pathways.

Acknowledgments

We apologize to authors whose primary research papers were not cited due to the format of this News and Views article.

Using host molecules to increase fungal virulence for biological control of insects

Comment on: Fan Y, et al. *Nat Biotechnol* 2012; 30:35–7; PMID:22231090;

<http://dx.doi.org/10.1038/nbt.2080> and Fan Y, et al. *PLoS One* 2012; 7:e26924; PMID:22238569;

<http://dx.doi.org/10.1371/journal.pone.0026924>

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<http://dx.doi.org/10.4161/viru.20956>

Unlike the situation for many fungal pathogens, i.e., those of most plants and animals, where efforts at understanding the pathogenic process are aimed toward controlling the disease-causing agent, research on entomopathogenic fungi seeks to develop new strategies for improving the ability of the fungus to target its insect hosts. Previous work has shown that fungal virulence can be increased via overexpression of insect cuticle-degrading enzymes, e.g., proteases and chitinases, and by expression of insect toxins derived from various organisms. A new approach in which insect derived molecules are used against the insects themselves within the context of the microbial pathogenic process has recently been described. Depending upon the host and molecule chosen it is theorized that target specific enhancement of virulence is achievable, a critical step in developing safer and more effective biopesticides. Exploiting host molecules also places a higher burden on potential resistance development as compared with use of chemical pesticides and/or insect toxins.

As an alternative to chemical pesticides, entomopathogenic fungi have long been

considered as potential candidates for insect pest control. The history of this research and any of its applications appears to have undergone periodic cycles of alternating intense and waning interest. In part, these cycles have been as a result of the development and introduction of cheaper and more effective chemical pesticides and the often less than satisfying performance of the fungal microbial agents in the field. The current status of the field appears to be one of cautious renewal of interest. The economic and industrial aspects of these efforts will not be considered here, but have been recently reviewed (Glare et al., *Trends Biotechnol* 2012). The major impetus driving this interest is the gradual banning of the use of chemical pesticides coupled to the lack of few new such chemicals in the near-term use and/or developmental stage. The two most widely studied entomopathogenic fungi are *Metarhizium anisopliae* and *Beauveria bassiana*, both Environmental Protection Agency (EPA, US, as well as by the respective EU regulatory agency)-approved insect biological control agents. Both organisms are currently used in various field applications and are available from a number of commercial sources

worldwide. However, while there are several notable instances of their successful use, a number of factors continue to limit practical field applications of mycoinsecticides. These include relatively low resistance to abiotic factors that can affect field use, i.e., low tolerance to high (> 37°C) and/or fluctuating temperatures, reduced efficacy at low humidity and low resistance to sunlight-derived UV irradiation. In addition, issues concerning the length of time to kill target insects remain since it can take from 3 to 15 d for the fungus to kill its insect targets. Finally, the need for high spore concentrations for effective suppression of target insect populations and (low) persistence issues place significant constraints on the ability to use spores and/or other fungal infectious propagules to cover large topographical areas, e.g., agricultural fields, in a cost-effective manner.

Recombinant DNA techniques have afforded an opportunity for strain improvement currently impossible. Attempts at targeted strain isolation and/or selection, parasexual crossing or protoplast fusion have resulted in only incremental increases in virulence and/or a desired trait. Genetic

engineering via addition of genes whether for pathogenicity determinants, stress resistance or some other factor that could increase the applicability of the fungal agents represents the most versatile approach at strain manipulation. The first such application targeted the (over) expression of an endogenous fungal protease that had been implicated in the degradation of insect cuticle substrates, i.e., facilitated the penetration of the host integument (St. Leger et al., Proc Natl Acad Sci U S A 1996). In this early work, although the median lethal concentration (LD_{50}) was unaffected, increased expression of the protease (Pr1) decreased the survival time (LT_{50}) by ~25%. Since then a variety of proteases, chitinases, the latter facilitating the degradation of the chitin polymer that constitutes the major carbohydrate constituent of the insect cuticle, and protease-chitinase hybrid proteins have been expressed in either *M. anisopliae* and/or *B. bassiana*, each showing some improvement in targeting of insect hosts.

A second, major development in the field occurred with the expression of an insect-specific scorpion neurotoxin in *M. anisopliae* which increased fungal virulence (i.e., decreased the LD_{50}) a dramatic 22-fold against the tobacco hornworm (*Manduca sexta*) and 9-fold against adult yellow fever mosquitoes (*Aedes aegypti*) (Wang and St. Leger, Nat Biotechnol 2007). This strain also resulted in a ~25–30% decrease in LT_{50} , thus killing insects faster and with fewer spores. Since then a number of other insect toxins have been expressed in either *M. anisopliae* or *B. bassiana* with similar results. This approach, however, has not been without controversy. Concerns regarding the use of strains expressing neurotoxins (even insect-specific) as well as the nature of the relatively non-specific enhancement of virulence that could potentially affect non-target or beneficial insects have been raised. More recently, the development of transgenic *M. anisopliae* that target the Plasmodium malarial parasite has been reported (Fang et al., Science 2011). In this approach, rather than increasing the virulence of the biological control agent, the fungus was engineered to express a single-chain antibody that agglutinates the sporozoite, thus reducing the parasite load in the mosquito and decreasing transmission of the disease causing agent. This development may be transformative in that it is likely to be the best current candidate for approval in field trials.

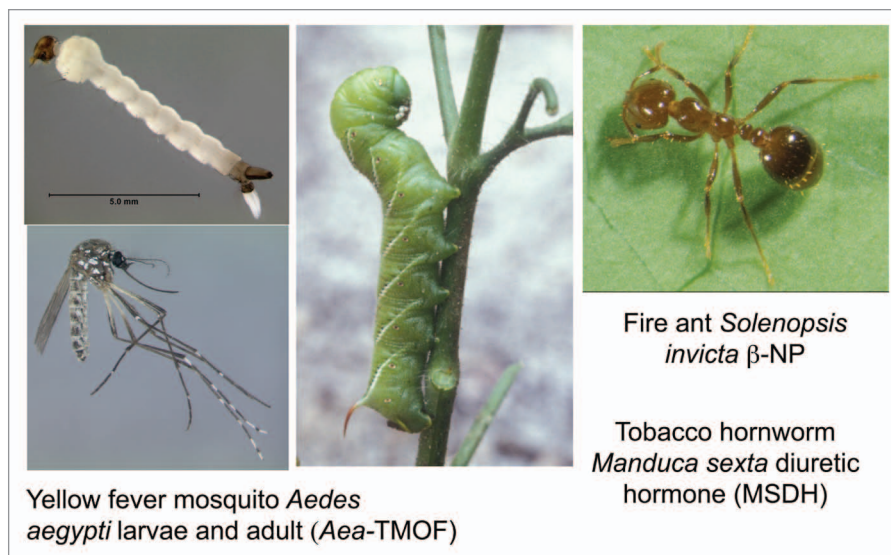


Figure 1. Using host molecules to improve the efficacy of fungal biological control agents. Various host-derived peptides, including the mosquito (*A. aegypti*) trypsin modulating oostatic factor (TMOF), the *Manduca sexta* diuretic hormone (MSDH) and the fire ant (*S. invicta*) β-neuropeptide (β-NP) were selected as candidate molecules for expression in a fungal-insect pathogen (*B. bassiana*) in order to determine whether these factors can be used against the insects themselves using the fungus as a means of delivering the compound. Images courtesy of C. Zettel, L. Buss, and Castner (University of Florida, Entomology and Nematology).

Given this context, we sought to expand the toolbox available for increasing the virulence of these fungi within a different conceptual framework. All insects (as well as most other organisms) use proteins and peptides to regulate critical physiological processes. These molecules are often regulated by strict developmental and tissue-specific mechanisms. We reasoned that disruption of the expression pattern of specific proteins/peptides via exogenous addition of the molecule could result in physiological consequences that would make the insect more susceptible to attack by microbial pathogens. Given the broad spectrum of molecules that would meet this description, several criteria were used to guide the selection of candidate molecules. First, evidence (in the literature) that the candidate molecule was essential for proper development or critical in key physiological and/or immune related processes. Second, evidence that the protein/peptide displayed some form of toxicity when administered to the insect. A third criteria, that significantly narrowed the field of potential candidates was evidence of field tests and/or agency approval of the molecule for actual use against insects. Finally, as targets for these efforts, we chose mosquitoes, which represent globally important vectors of disease causing agents, and fire ants, an invasive pest species

that results in significant agricultural, ecological and hence economic losses, which also have important human health-related impacts.

One of the most promising candidates identified was the trypsin-modulating oostatic factor (TMOF) derived from the mosquito, *Aedes aegypti* (Fig. 1). TMOFs are hexa- and deca-peptides that terminate trypsin biosynthesis in the insect gut and are required for normal development and ovipositing. TMOF circulates in the hemolymph, binding to receptors on the hemolymph side of the gut and inhibits trypsin biosynthesis via translational control of trypsin mRNA. TMOF resists proteolysis in the gut and can traverse the gut epithelium to enter into the hemolymph. Due to this property, it was shown that exogenous feeding of *Aea*-TMOF to mosquitoes blocks their ability to digest food, bloodmeal or otherwise, subsequently resulting in starvation and death in both adults and larvae. A TMOF ortholog was also identified from the gray flesh fly, *Sarcophaga bullata*. The two TMOF sequences are highly divergent with the *A. aegypti* sequence corresponding to 10 amino acids: YDPAPPPPPP, and the *S. bullata* TMOF to six amino acids: NPTNLH. TMOFs therefore appear to be good candidates regarding specificity with minimal non-target effects, a key factor with respect to the desire

to develop target specific agents. Commercial production and use of *Aea*-TMOF is currently under development, however, the most significant obstacle to practical application of the peptide is the lack of a mechanism by which the peptide can be delivered to the mosquito. Since *B. bassiana* is already a pathogen of mosquitoes, we hypothesized that expression of *Aea*-TMOF in the fungus would represent an approach at delivering the peptide to the target, while increasing the virulence of the biological control agent in a host specific manner.

As further proof-of-concept two additional insect-derived candidate molecules were selected for expression in *B. bassiana*: the tobacco hornworm, *Manduca sexta*, diuretic hormone (MSDH) and the fire ant, *Solenopsis invicta*, β -neuropeptide (β -NP). Insect diuretic hormones regulate fluid homeostasis and MSDH is a member of the corticotropin-releasing factor-related family of peptides. These peptides act upon insect Malpighian tubules, excretory organs that lie in the abdominal cavity and empty into the junction between the midgut and hindgut, affecting their secretion of water and solutes. Administration of synthetic MSDH to insects results in fluid loss through the gut and epidermis, decreased feeding, and ultimately leads to the death of the organism. β -NP is a member of the pyrokinin/pheromone biosynthesis activating neuropeptide (PBAN) family that is characterized by the presence of a C-terminal FXRRL-NH₂ sequence. β -NP is often (co-transcribed and) co-translated into a larger protein that is subsequently post-translationally cleaved to yield smaller peptides. These smaller peptides can include diapause hormone (DH), three peptides termed α , β and γ -neuropeptides and PBAN itself, the latter of which is often found between the β - and γ -neuropeptides. All of the peptides contain the C-terminal FXRRL-NH₂ motif, with the amidation reaction representing a subsequent (after cleavage) post-translational modification of the peptides. Depending upon the insect species, these peptides are thought to function in a wide range of physiological processes that includes stimulation of pheromone biosynthesis, induction and/or termination of diapause, pupariation, melanization and potentially mediation of defense responses. In *S. invicta*, the α -peptide is lacking.

In all, we assessed the impact of expressing *Aea*-TMOF, *Sb*-TMOF, MSDH and *Si*- β -NP in the fungal insect pathogen *B. bassiana* (Fan et al.,

Nat Biotechnol 2012; Fan et al., PLoS One 2012). Each peptide was independently expressed in constructed fungal strains via transformation using vectors containing a constitutive *B. bassiana* derived *gpd* promoter and the nucleotide sequences of each peptide fused to a signal sequence to drive extracellular secretion of the molecule produced. Production of the peptides in culture media was confirmed by mass spectrometry and the concentrations ranged from 0.2 to 1.0 μ M. Expression of MSDH increased the virulence (i.e., decreased the lethal dose, LD₅₀) from 5- to 10-fold toward Lepidopteran hosts including *M. sexta* and the greater waxmoth, *Galleria mellonella*, as well as toward *A. aegypti*. In addition, the time to death (LT₅₀) decreased by ~25–35% as compared with the wild-type parental strain. These data suggest that MSDH would act as a broad host-range targeting molecule.

Similarly, expression of *Aea*-TMOF in *B. bassiana* decreased the LD₅₀ 6- to 7-fold and decreased the LT₅₀ by ~30% against blood-fed female mosquitoes. However, *Aea*-TMOF expressing strains were no more effective against flesh flies (*S. bullata*) than the wild-type parent, and expression of *Sb*-TMOF did not increase virulence toward mosquitoes. Interestingly, expression of *Sb*-TMOF also did not increase virulence toward *S. bullata*, suggesting that the physiological requirement and/or role of TMOF in the flesh fly may differ significantly from that in mosquitoes. In addition to increasing virulence toward both the adult and larval stages of the mosquito, several important “side effect” impacts were noted for the *B. bassiana* strain expressing *Aea*-TMOF. As expected, gut trypsin activity was inhibited, but larval development was stunted and fecundity was dramatically decreased, with female mosquitoes laying ~55% fewer eggs than uninfected controls. Since one of the major obstacles regarding field use of fungi against mosquitoes is that the relative time frame it takes to kill the insect would still allow for a reproductive cycle, the decreased fecundity effect could have a significant impact on biological control efforts that seek to decrease vector populations and hence disease transmission.

Expression of *Si*- β -NP in *B. bassiana* increased fungal virulence approximately 6-fold toward fire ants (decreased LD₅₀) and decreased the mean survival time (LT₅₀) by ~30%. In this instance no significant differences were seen between the wild-type strain

and the *Si*- β -NP expressing strain toward the Lepidopteran hosts *Galleria mellonella* and *Manduca sexta*. However, (unexpectedly) alterations in social behavior were noted in ants infected with the *Si*- β -NP expressing strain that did not occur during infections using the wild-type *B. bassiana* parental strain. Whereas under normal conditions as well as during infection by the wild-type *B. bassiana* strain, the fire ants would remove their dead from the surrounding areas of their nests, forming discrete “bone piles” or “cemeteries,” a phenomenon termed necrophoretic behavior, ants infected with *Si*- β -NP appeared to leave their dead conspecifics randomly distributed in the assay chambers. Necrophoretic behavior along with grooming represent two important behavioral adaptations that have limited the use of biological control agents in targeting fire ants and other social insect pests, since these actions help minimize the spread of infections throughout a colony. It remains to be seen whether the observations made in the laboratory would have any real impact in the field; however, these results do illustrate that insect pest behaviors can potentially be modulated in a target specific manner.

Although we have used fungi as the vehicle to deliver host molecules to an intended insect target, the use of insect derived proteins and peptides could potentially be expanded to other insect biological control agents such as viruses, bacteria and nematodes. There are several potential advantages to the approach outlined here that although require further investigation and verification, should be noted. First, appropriate selection of the host molecule can lead to target specific increases in virulence. Second, additional beneficial (in the sense of increasing the impact of the biological control agent) can be incorporated in the design selection, i.e., via selection of molecules that disrupt fecundity, feeding or other processes and/or behaviors. Finally, we theorize that the development of resistance to our approach is minimized as the host molecules (peptides) selected participate in critical or ideally essential processes that are species and tissue specific. Contrary to the use of chemical pesticides and/or toxins, where often a single mutation could result in resistance, mutations that might arise and could compensate for the dose given by the fungal-expressed product during infection would result in severe developmental defects, particular since the fungus remains a pathogen even in the absence of

the expressed molecule. We speculate that this would represent a steep fitness cost greater than the potential development of resistance

to a pesticide or toxin. Furthermore, multiple host molecules and/or combinations with other pathogenicity/targeting factors can be

expressed in the same fungal strain to further improve the efficacy and safety of biologically based pesticides.

Can high-pathogenic avian influenza viruses with novel hemagglutinin serotypes other than H5 or H7 emerge?

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Highly pathogenic avian influenza viruses (HPAIV) cause devastating outbreaks in domestic poultry worldwide. Moreover, they proved to be responsible for repeated severe human infections with high lethality raising concerns about their pandemic potential. HPAIV are restricted to the hemagglutinin serotypes H5 and H7 and evolve from low-pathogenic precursors. Since the introduction of an artificial polybasic HA cleavage site into non H5/H7 HA of low-pathogenic strains may result in high-pathogenic viruses, the emergence of HPAIV with novel serotypes is conceivable. However, the observed HA serotype restriction in “natural” HPAIV indicates a unique predisposition of the H5/H7 HA for an insertion mutation coding for a polybasic hemagglutinin cleavage site.

Highly pathogenic avian influenza viruses (HPAIV), formerly known as fowl plague viruses, cause devastating losses in domestic poultry. Within the past 130 years, more than 30 outbreaks have been reported worldwide. Furthermore, repeated severe HPAIV infections in humans with high lethality have raised concerns about adaptation to the human host sparking a novel pandemic. An essential step in the replication cycle of all influenza A viruses is the cleavage of the hemagglutinin (HA) precursor HA0 into the HA1 and HA2 fragments. Only the cleaved HA can undergo a conformational change in the acidic endosome resulting in exposure of the hydrophobic N termini of the HA2 fragments (the fusion peptides) to mediate fusion of the virion envelope with the endosomal membrane. This proteolytic activation requires host proteases that recognize specific cleavage sites. The common HA cleavage site (HACS) contains a monobasic motif that is prevalent in all human, mammalian and low-pathogenic avian influenza viruses (LPAIV). In contrast, all HPAIV carry a polybasic HACS. This difference in cleavage motifs leads to the recruitment of different host proteases. The

monobasic HACS is cleaved by tissue-restricted proteases such as TMPRSS2, HAT, other trypsin-like and perhaps yet unidentified proteases, whereas the polybasic HACS is processed by the ubiquitous proprotein convertase furin and related subtilases. This utilization of different proteases results in distinct tissue tropisms. Whereas LPAIV and human influenza viruses are mostly confined to the digestive or respiratory tracts, respectively, the HPAIV display a very broad organ tropism affecting several organs including the central nervous system, often considered the prime cause of death in infected birds. Since experimental removal of the polybasic HACS invariably resulted in a virus with low-pathogenic phenotype, it is considered the key virulence determinant of HPAIV.

All HPAIV have evolved from low-pathogenic precursors due to extension of the HACS by an insertion mutation of the HA gene. Remarkably, different amino acid sequences at the HACS indicate independent mutation events. For a few HPAIV, the insert was found to originate from recombination with viral (NP or M gene) or host (ribosomal 28S) RNA, or was suggested to result from polymerase slippage; however, in most strains, the mechanism for insertion mutation has remained unknown.

Beside the polybasic HACS, HPAIV differ from LPAIV in that they are restricted to the HA serotypes H5 and H7, two of 16 serotypes known in avian viruses. Since the reason for this serotype restriction is unclear, we aimed to distinguish between the compatibility of a polybasic cleavage site with H5/H7 HA only and a unique predisposition of those two serotypes for insertion mutations. Artificial introduction of a polybasic HACS into low-pathogenic strains of HA serotypes H3, H4 or H9 did not lead to a high-pathogenic phenotype, in contrast to an HACS mutant of an H6N1 virus. Remarkably, a polybasic cleavage site mutant of a low-pathogenic H5N1

virus did not display high virulence either. Reassortants carrying the HA gene from an H5 HPAIV with the other seven genes originating from either the H9 or H5 LPAIV showed only temporary non-lethal disease in chicken, whereas reassortants with the reciprocal gene constellation, i.e., the LPAIV H9 or H5 HA with artificial polybasic HACS and the genetic background from an HPAIV, were lethal for chickens. Therefore, virulence determinants of HPAIV exist beyond the essential polybasic HACS and reside both in the HA and the other gene segments.

To investigate whether HPAIV of other serotypes could emerge in the genetic background of an HPAIV, we introduced a polybasic cleavage site into the HA of LPAIV with serotypes H1, H2, H3, H4, H6, H8, H10, H11, H14 or H15, and rescued HA reassortants after co-transfection with the genes from either a low-pathogenic H9N2 or high-pathogenic H5N1 avian strain. Those recombinants which contained the engineered H2, H4, H8 or H14 genes in the HPAIV background, were lethal in chickens and exhibited intravenous pathogenicity indices of 2.79, 2.37, 2.85 and 2.61, respectively, equivalent to naturally occurring H5 or H7 HPAIV. Thus, in the presence of a polybasic HACS, nonH5/H7 HA can support a highly pathogenic phenotype in the appropriate viral background, indicating requirement for further adaptation. Hence, the observed restriction of natural HPAIV to serotypes H5 and H7 is likely due to their unique predisposition for acquisition of a polybasic HACS.

In the light of our finding that HPAIV with non-H5/H7 serotypes can be generated experimentally, it is conceivable that HPAIV with novel serotypes could emerge in the future. H5N1 HPAIV have become endemic in several regions such as Southeast Asia or Egypt yielding ample opportunity for reassortment with an LPAIV to evolve into an HPAIV. However, the molecular

basis for the emergence of an HPAIV is still not clear and may include these three preconditions: the HA has to be prone to acquire a polybasic HACS, a lowered optimal pH to trigger fusion of the HA has to be achieved, and other virulence and host range determinants like the NA stalk deletion have to be established. Furthermore, it is still uncertain whether such additional virulence determinants have to be accumulated before or after formation of the polybasic HACS. Moreover, in which avian host does this process take place? This evolutionary

step is likely to happen in gallinaceous poultry prior to an HPAI outbreak as suggested by previous observations. For instance, prolonged circulation of low-pathogenic precursor viruses in chickens was found for several months before the outbreaks in Mexico 1994 and Italy 1999. Therefore, at least some of the virulence or host range determinants might be required for virus survival in the chicken flocks prior to acquisition of the polybasic HACS by an insertion mutation that occurs at considerable lower probabilities than point mutations.

Taken together, the natural formation of a polybasic HACS has so far been restricted to H5 or H7 LPAIV. This HA serotype restriction observed in authentic HPAIV points to a unique predisposition of the H5/H7 HA for polybasic HACS formation. Since in presence of the required virulence determinants in the HA and other genes, the experimental introduction of a polybasic HACS may result in high-pathogenic viruses with HA serotypes other than H5 or H7, the emergence of HPAIV with novel serotypes is conceivable.