Viral proteins and Src family kinases: Mechanisms of pathogenicity from a “liaison dangereuse”

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Abstract
To complete their life cycle and spread, viruses interfere with and gain control of diverse cellular processes, this most often occurring through interaction between viral proteins (VPs) and resident protein partners. Among the latter, Src family kinases (SFKs), a class of non-receptor tyrosine kinases that contributes to the conversion of extracellular signals into intracellular signaling cascades and is involved in virtually all cellular processes, have recently emerged as critical mediators between the cell’s infrastructure and the viral demands. In this scenario, structural or ex novo synthesized VPs are able to bind to the different domains of these enzymes through specific short linear motifs present along their sequences. Proline-rich motifs displaying the conserved minimal consensus PxxP and recognizing the SFK Src homology (SH)3 domain constitute a cardinal signature for the formation of multiprotein complexes and this interaction may promote phosphorylation of VPs by SFKs, thus creating phosphotyrosine motifs that become a docking site for the SH2 domains of SFKs or other SH2 domain-bearing signaling molecules. Importantly, the formation of these assemblies also results in a change in the activity and/or location of SFKs, and these events are critical in perturbing key signaling pathways so that viruses can utilize the cell’s machinery to their own benefit. In the light of these observations, although VPs as such, especially those with enzyme activity, are still regarded as valuable targets for therapeutic strategies, multiprotein complexes composed of viral and host cell proteins are increasingly becoming objects of investigation with a view to deeply characterize the structural aspects that favor their formation and to develop new compounds able to contrast viral diseases in an alternative manner.

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Key words: Interaction; Phosphotyrosine; Proline-rich motif; Src homology 2 domain; Src homology 3 domain

INTRODUCTION
Although not strictly meeting the definition of living organisms, viruses can replicate and spread, provided that they infect host cells to produce a new offspring. In this regard, viruses are obligate intracellular parasites that have evolved complex strategies to complete their life cycle, consisting of contrasting and even evading innate defense mechanisms of the host cell for which they have a tropism so as to take control over key cell pathways [1,2]. Therefore, they interfere with diverse cellular processes, especially those involving replication of genetic material, protein translation and trafficking, with viral structural and non-structural proteins undergoing post-translational modifications, such as phosphorylation, ubiquitination, glycosylation and cleavage prior to or after interacting with a vast part of
the host’s proteome, thereby forming new multimeric assemblies. Indeed, much effort has been made over the last few decades to dissect the cellular signaling pathways hijacked by viruses and the underlying molecular mechanisms, leading to the identification of several host cell factors playing a major role in the viral life cycle. Among these, Src family kinases (SFKs), a class of non-receptor tyrosine kinases comprising of eight members (Src, Yes, Fyn, Fgr, Lyn, Hck, Lck and Blk), have emerged as critical mediators between the cell infrastructure and the viral demands. Although traditionally known as molecular switches situated right beneath the plasma membrane, from there relaying extracellular cues and governing signal transduction, SFKs localize to virtually all the other cell compartments, thereby regulating a wide spectrum of cellular processes, such as growth, viability, cell cycle and metabolism. To gain a deeper understanding of how SFKs exert their function and how viruses can benefit from the interplay with SFKs, it is necessary to bear in mind the complexity of the multidomain organization and the mechanisms of activation of SFKs themselves. From the N- to the C-terminus, their structure consists of: (1) the Src homology (SH) 4 domain, a unique region that becomes myristoylated and/or palmitoylated for membrane association of SFKs; (2) the SH3 domain, which binds specific proline-rich motifs (PRMs); (3) the SH2 domain, which recognizes phosphotyrosine motifs; (4) the SH2-kinase linker, (5) a catalytic SH1 domain; and followed by (6) a C-terminal tail implicated in the downregulation of SFKs (Figure 1). The activity of SFKs is mainly modulated by the phosphorylation state of 2 critical tyrosine residues, Tyr416 and Tyr527 (based on the amino acid numbering of chicken c-Src and corresponding to Tyr419 and Tyr530 in humans), with opposing effects: the former, which lies in the activation loop, is subjected to autophosphorylation when the SFK is activated, whereas the latter is targeted by C-Src tyrosine kinase, resulting in the inactivation of the tyrosine kinase. The latter event is induced by a closed conformation of the SFK through 2 major intramolecular inhibitory interactions, binding of the C-terminal phosphotyrosine (Tyr527) itself to the SH2 domain and interaction of a polypoline type II helical motif (PP II) in the SH2-kinase linker with the SH3 domain. On the other hand, multiple events can induce disruption of such inhibitory mechanisms, such as dephosphorylation of the tail, with its displacement from the SH2 domain and/or displacement of the PP II motif from the SH3 domain, ultimately resulting in the full activation of SFKs. All these features, either functional or structural, can be exploited one at a time or in combination by most, if not all, viruses to take over the cell machinery, from the cell entry, all through the genome replication until the release of new particles. As to internalization, viral particles induce, upon interaction of cognate membrane receptors, activation of SFKs, which, as apical cellular transducers in receptor-mediated cellular signaling, take part in the activation of clathrin-, caveolin-dependent endocytic pathways, or the alternate mechanism based upon macropinocytosis and particle assembly and spread.

This review offers a brief summary of the current knowledge of the molecular mechanisms underlying the interactions between SFKs and VPs, as well as the consequences thereof, so as to highlight common or rather specific structural motifs that might become molecular targets for disrupting such associations and to provide a new perspective in managing viral infections.

INTRACELLULAR VIRAL HIJACKING OF SFKs

It has been largely described that a plethora of viruses, once they enter the cell, utilize SFKs to foster the different steps of the viral life cycle, although the molecular mechanisms have not been elucidated in many cases. However, accumulating evidence indicates that direct interaction of VPs with SFKs results in the activation of SFKs with subsequent (1) phosphorylation of VPs, which acquire new functional properties (Figure 2A-C), or (2) deactivation of SFKs to cell compartments targeted by specific VPs, where SFKs can exert their catalytic and non-catalytic action (Figure 2D), all these events often being well intertwined with one another. Although involved as apical molecular switches orchestrating virtually all cellular signaling pathways and sharing a highly similar structural arrangement, the single SFKs often demonstrate a differentiated responsiveness to VPs, which depends on the inbuilt properties of the kinase structure and the mode of interaction with VPs, especially with respect to the SH3 and SH2 domains, resulting in remarkably
SH2
HP-1
Phosphorylation sites (tyrosines)
Y250, Y315 and Y322

Virus
Hepatitis C virus
VP-2
C-terminus containing
C-t

VP-1
VP
None
VP

Src
pY-VP
VP
None
Y66, Y69 and Y71

Y114 and Y127

Human T-cell leukemia virus type 1
None
VP

HP-2 (SFKs)
HP-2 (SFKs)
Y not identified
None
VP

SH1
Src and Fyn
Y290, Y315 and Y322

Lck
Y114 and Y1127

Lck
Y not identified

Fyn
Y66, Y69 and Y71

Table 1  List of the viral proteins described in this review

| Protein                                      | Virus                        | Src family kinase | Phosphorylation sites (tyrosines) |
|----------------------------------------------|------------------------------|-------------------|-----------------------------------|
| Polyoma middle-T antigen                     | Polyomavirus                 | Src and Fyn       | Y290, Y315 and Y322               |
| Tyrosine kinase interacting protein          | Herpesvirus saimiri          | Lck               | Y114 and Y1127                    |
| Tegument protein VP11/12                    | Herpes simplex virus         | Lck               | Y not identified                  |
| Accessory viral protein X                   | Human immunodeficiency viruses and simian immunodeficiency viruses | Fyn           | Y66, Y69 and Y71                   |
| Latent membrane protein 2A                  | Epstein-Barr virus           | Lyn               | Y74 and Y85                        |
| Non-structural protein 5A                    | Hepatitis C virus            | Src               | Y not identified                   |
| Accessory protein Nef                       | Human immunodeficiency virus-1| SFKs             | None                              |
| Non-structural 1                            | Avian influenza virus        | Src               | None                              |
| Accessory protein p13                       | Human T-cell leukemia virus type 1 | SFKs       | None                              |
| RNA-dependent RNA polymerase                | Hepatitis C virus            | Src               | None                              |
| non-structural protein 5B                   |                              |                   |                                   |

SFKs: Src family kinases; VP: Viral protein.

Figure 1  Diagram representing the domain organization of Src family kinases. As reported in the text, the C-terminus (C-t in the figure) when phosphorylated at Tyr527 binds to the Src homology (SH)2 domain and the polyproline type II helical motif (PP II) motif in the SH2 kinase linker (SH2-KL in the figure) engages the SH3 domain, thus inducing an inactive conformation. Disruption of these inhibitory interactions, in the case of viruses mostly induced by proteins bearing tyrosine phosphorylated or proline-rich motifs, leads to the full activation of Src family kinases.

VPS AS SUBSTRATES FOR SFKs

The number of tyrosine phosphorylated VPs is, for the time being, rather small and these mostly include molecules from classes of viruses that can establish chronic infections, such as herpesviridae, polyomaviridae and retroviridae, among others (Table 1).

The polyoma middle-T (MT) antigen, an early product of the viral lytic cycle of the polyomavirus, which is known to contribute to the onset of multiple tumors, is phosphorylated by Src and Fyn at tyrosines that become docking sites for molecules essential for downstream signaling [e.g., PLCγ, phosphatidylinositol 3-kinase (PI3K) and Shc][11]. This multiprotein complex then mimics a...
constantly activated growth factor receptor at the plasma membrane. Importantly, a preliminary step for SFKs to phosphorylate the MT is their activation, which occurs upon binding of their SH1 domain to the MT itself and concurrently to the dimeric core of the serine/threonine protein phosphatase 2A, which acts as a scaffold between the MT and SFKs\(^\text{[22,23]}\) (modeled in Figure 2A).

Another VP that associates to the plasma membrane and exhibits a transforming ability owing to phosphorylation by the SFK Lck is the tyrosine kinase interacting protein (Tip), an oncoprotein encoded by the genome of herpesvirus saimini, a T-lymphotropic monkey herpes virus. Importantly, Lck phosphorylates Tip at different tyrosines responsible for binding the SH2 domains of various signaling molecules, among which are Lck itself, STAT3 and STAT6, all required for promoting the transformed phenotype\(^\text{[14,15]}\). Also in this case, the essential requirement for phosphorylation is the initial interaction between the SFK and the substrate, here mediated by Tip’s sequence homologous to the C-terminus of SFKs and SH3-binding PRM with Lck\(^\text{[16]}\). The plasma membrane also serves as the anchoring site for VP11/12, a tegument protein residing between the capsid and the envelope of the herpes simplex virus immediately after viral entry, this VP being strongly phosphorylated by SFKs and hence modulating cellular signaling pathways\(^\text{[17]}\). Of note, the function of VP11/12 as a component of a signalosome differs from that exerted by VP11/12 itself in the virion assembly at the perinuclear cytoplasmic foci of the infected cells, whereby it also massively localizes. During T cell infection, VP11/12 behaves as an activator and a substrate of Lck, which phosphorylates VP11/12 at a number of tyrosines serving as docking sites for, at least as documented thus far, the SH2 domain of Lck itself and the PI3K regulatory subunit p85\(^\text{[18]}\). It is to be underlined that under these conditions Lck does not operate as a transducer of TCR signaling but triggers cellular processes peculiarly directed by VP11/12, which are still being investigated. Other aspects that remain to be explored are the mechanism of Lck activation and the mode of recognition of VP11/12 as a substrate by Lck, for which, on the basis of the examples mentioned above, an interaction between linear binding motifs in VP11/12 and the non-catalytic domains of the SFK may be required. Indeed, the primary sequence of VP11/12 exhibits several PRMs, which might provide an anchorage for the SFK-SH3 domain with subsequent structural changes from the inactive close to the active open conformation of SFKs, to the catalytic pocket of which the access of VP11/12 as substrate is then favored. That the phosphorylation of VPs and the interaction between specific motifs harbored by VPs and the modular domains of SFKs may be connected seems to be further confirmed by the findings regarding Vpx, an accessory protein of the human immunodeficiency virus type 2 (HIV-2) and the simian immunodeficiency virus\(^\text{[19]}\). This VP, which is coupled to the preintegration complex and localizes to the nuclei of infected cells, shuttles to the cytoplasm to be incorporated into the viral core and ensures efficient viral replication only if it becomes phosphorylated by the SFK Fyn (especially at Y66, 69 and Y71)\(^\text{[20]}\). This event is made possible by a preliminary interaction between Vpx PRMs and Fyn SH3 domain, which may bring Vpx to the Fyn catalytic domain\(^\text{[21]}\).

The nature of the binding of other VPs that are tyrosine phosphorylated and bind to SFKs remains unclear, requiring in-depth examination. One instance may be latent membrane protein 2A (LMP2A), a VP encoded by Epstein-Barr virus genome in infected B cells, which, by sequestering proteins normally associated with the B cell receptor (BCR) in the absence of BCR-triggering antigens, mimics the latter with subsequent activation of downstream survival signaling pathways, hence altering normal B cell development\(^\text{[22]}\). In particular, it has recently ascertained that phosphorylation of LMP2A by Lyn, the most expressed SFK in B lymphocytes, exerts a dual role, namely as (1) an early requirement for the formation of the LMP2A-based signalosome through creation of docking sites for the Spleen tyrosine kinase (Syk), the SH2 domain-containing adapter protein B (Shb) and Lyn itself\(^\text{[23]}\), and as (2) a key regulatory event in the modulation of LMP2A-dependent signaling by degradation of various components of this multiprotein complex (Lyn and even LMP2A itself)\(^\text{[24]}\). Also in the case of LMP2A, it is not clear how Lyn becomes activated before phosphorylating the VP. In this respect, of the several PXXP motifs along the sequence of LMP2A, the N-terminal ones have only been tested for binding SH3 domains, proving unable to do so and thus leaving open the possibility that the C-terminal PPⅡ helical motif may be a site of interaction and activation for Lyn\(^\text{[25]}\) (the basic mechanisms demonstrated or thought to underlie interaction and downstream effects of Tip, VP11/12, Vpx and LMP2A with SFKs are illustrated in Figure 2B).

An even more intricate case is represented by the non-structural protein 5A (NS5A) encoded by the genome of the hepatitis C virus (HCV), which is essential for HCV replication and virion assembly in hepatocytes\(^\text{[26]}\). This VP has recently been found to be phosphorylated at tyrosines within SH2-binding motifs, in addition to being highly phosphorylated at serine residues\(^\text{[26-28]}\). It also bears a conserved C-terminal PRM that has been shown to bind to the recombinant SH3 domains of Hck, Lck, Lyn and Fyn, but not Src, negatively affecting the activity of Hck, Lck and Lyn but stimulating that of Fyn\(^\text{[29]}\). Although not binding to Src via the SH3 domain thereof, NS5A interacts with the SH2 domain of this SFK after being phosphorylated in HCV-infected hepatocytes, an event which is critical for viral replication, and whereby Fyn, the only SFK activated by NS5A upon interaction mediated via SH3 domain, proves dispensable\(^\text{[27]}\). A possible mechanism for the phosphorylation of NS5A and subsequent SH2 domain-mediated interaction with Src is set forth in the next section and modeled in Figure 2C. Besides, in B cells, whereby infection by HCV causes mixed cryoglobulinemia and B cell non-Hodgkin’s lymphoma, Fyn inter-
acts with NS5A through both its SH2 and SH3 domains in a tyrosine phosphorylation-dependent manner and by recognition of a PRM of NS5A itself, respectively, resulting in inhibition of viral replication in parallel with Fyn enhanced activity[28]. These data again confirm that VPs need to specifically select SFKs, whose non-redundant functions can be exploited to dictate the different steps of viral replication.

EFFECTS ON LOCATION AND ACTIVITY OF SFKS MEDIATED BY INTERACTION WITH VPS

The mechanisms leading to the phosphorylation of VPs described above seem to mainly point to the earlier binding of VPs to the SH3 domain of SFKs as the activation event for SFKs themselves, the newly phosphorylated tyrosines providing an anchorage for signaling molecules utilized by the virus for its own benefit, even for SFKs among others. We shall hereafter illustrate a set of VPs that are still able to interact with the SH3 domain of SFKs and function as activators of SFKs without being their substrates (a general diagram is shown in Figure 2D). From this list, the well-characterized HIV-1 accessory protein Nef stands out, it being essential for virus replication and acquired immunodeficiency syndrome pathogenesis by interacting with various host cell proteins involved in immune recognition and survival, among which SFKs are targeted with high selectivity[30-32]. Among the many interaction motifs along its sequence, Nef harbors a highly conserved PxxPxR motif, which, together with a hydrophobic pocket in the core region[33], takes part in the interaction between Nef and the SH3 domain of a few of SFKs, namely Hck and Lyn[33], thus causing disruption of the negative regulatory interaction between the SH2-kinase linker and the SH3 domain itself on the back of the kinase domain and subsequent activation. Of note, in spite of the high conservation of Nef’s regions for binding to these SFKs, other determinants have emerged as critical in their substrates (a general diagram is shown in Figure 2D).

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The non-structural 1 (NS1) protein of the avian influenza virus (AIV), a multifunctional protein with interferon-antagonistic properties, is a further example of the interaction between the SH3 domain of SFKs through PRMs of specific VPs as a means to mediate pathogenicity by viruses[38]. First being isolated in poultry and having exhibited high virulence and pathogenicity, it was also shown to cross the species barrier, involving human fatalities, especially in the Far East. The possibility that such a viral strain or new reassortants might cause severe pandemics generated a new interest in evaluating the pathogenicity determinant in the spread and pathogenesis of the disease[39,40]. NS1 possesses two functional domains, the N-terminal RNA-binding domain containing one nuclear localization signal as well as a SH2- binding motif targetable after phosphorylation of the tyrosine residue, and the C-terminal effector domain, with two PRMs, a further nuclear localization sequence and a PDZ binding motif. Of the PRMs, the first is generally conserved in all influenza genotypes and harbors the structural determinants for binding the SH3 domain of the PI3K regulatory subunit p85 (P/LPxPP)[41], whereas the C-terminal has a certain variability that parallels its capability of interacting with the SH3 domain of and activating the SFK Src[42]. In this respect, only NS1 bearing the consensus sequence type 2 for binding SH3 domains of Src (PXXPXK/R), was able to enhance the SFK activity, which occurred in virus genotypes that caused the most severe human influenza pandemics in 1918 and killed turkeys in Italy in 1999 with heavy economic losses, whereas the viral strains that were mutated in this region did not affect SFK activity. To date, the function of an activated form of Src in AIV-infected cells is not fully clear, although it is thought that it may be related to the localization of the Src-NS1 complex.

Another prototype for the change in subcellular localization of SFKs induced by VPs is represented by the human T-cell leukemia virus type 1 accessory protein p13, although the data refer to experimental approaches in vitro or in cultured cells transfected with the single SFKs or p13 itself. p13 is known to localize to mitochondria thanks to its N-terminal mitochondrial localization signal, where it brings about an inward K+ current across the inner mitochondrial membrane, leading to swelling, depolarization and increased respiratory chain activity[43]. Recently, p13 has been found to bind to the SH3 domain of SFKs by a well-defined C-terminal PRM and to act as a carrier for SFKs themselves into mitochondria, this new localization of SFKs resulting in (1) a sharp rise in intramitochondrial tyrosine phosphorylation; and (2) a significant mitigation of p13’s aforementioned effects on mitochondria[44]. This observation seems to be in line with recent findings that strongly suggest a new role for SFKs as factors that help preserve mitochondrial structural and functional integrity under stressful conditions[45], thus further providing
novel insights into the catalytic and non-catalytic role of SFKs in virus-infected cells. The complex relationship between such functions of SFKs and the physical interaction thereof with VPs fits into another model represented by the events that lead to the formation of the HCV replication complex, in which the RNA-dependent RNA polymerase NS5B and the above mentioned substrate for SFKs, NS5A, together with the SFK Src, take part. The integrity of this multimolecular complex, which is essential in HCV replication, requires Src as a scaffold for promoting a tighter interaction between NS5A and NS5B, with both the modular SH2 and SH3 domains as well as the catalytic activity of Src being implicated. Interestingly, Src is the only SFK that does not bind to NS5A through its SH3 domain, whereas it recognizes the C-terminal proline-rich region containing a non-canonical SH3 binding motif within NS5B. On the other hand, the SH2 domain of Src interacts with a yet-to-be-identified tyrosine phosphorylated binding motif within NS5A, respectively. Although the molecular mechanism underlying the formation of the complex is unknown, it is tempting to speculate that Src stabilizes the weak interaction between NS5A and NS5B by firstly recognizing the PRM of NS5B, although it does not display an optimal consensus for binding to the SH3 domain of SFKs, thus becoming activated and enabled to phosphorylate NS5A. Phosphotyrosines on NS5A can then be targeted as docking sites by Src, further strengthening the stability of the heterotrimer (Figure 2C). What we hereby again underscore is that VPs and cellular proteins, such as SFKs, complexed into a new operative unit can serve as a key to interpreting the intricate relationship between host cells and viruses in order to elaborate novel strategies to disrupt aberrant multiprotein associations.

CONCLUDING REMARKS AND PERSPECTIVES

The significance of SFKs as critical mediators in the life cycle of viruses has been widely shown by the effects of the inhibition of their enzymatic activity or of their expression as well as by those related to the interaction with specific VPs, as hereby briefly reviewed. Indeed, this latter issue has become a new important field of investigation, with great efforts aimed at dissecting the structural aspects that favor such interactions, in order to develop therapeutic strategies capable of disrupting them to hamper viral replication. Although the different domains of SFKs exhibit various potential anchorage sites for VPs, including the SH2 and the catalytic domain, these preferentially target the SH3 domain of SFKs by their PRMs (usually a class II motif), which generally displays the consensus sequence PxΦPSxK/R (where Φ stands for a hydrophobic residue). The PRM-bearing protein can then compete with and displace the PP II within the SH2-kinase linker of SFKs, thus directly interacting with the SH3 domain and inducing the transition from the “closed” to the “open” conformation, with three possible outcomes: (1) altered localization of SFKs; (2) phosphorylation of the VP with generation of docking sites available for further interactions; and (3) hyperactivation of the kinase activity. This latter effect has been explained for Hck bound to Nef, but not thus far for other SFKs, through kinetic studies, which have highlighted that Nef’s PRM induces a change in the conformation of the active site of Hck by an allosteric mechanism with a decrease in the Km for ATP. Of note, even if all SFKs share a common domain structure and a mode of regulation, VPs may display different abilities to interact with each single SFK and to affect their catalytic activity by their PRM. This wide variability in the response of the single SFKs demands a further effort to extend our knowledge on the structural determinants of the SH3 domain also outside of the interface binding the viral PRM, this possibly providing the ground for the prediction of recognition elements. From a therapeutic perspective, two main schools of thought have surfaced in addressing this issue, one arguing for the use of bioengineered polypeptides capable of interfering with SH3 binding of SFKs to VPs and the other supporting the implementation of non-toxic kinase inhibitors that bind the catalytic groove of SFKs only if the VPSFK complex is formed (e.g., Nef:Hck). This latter approach would be remarkably useful in preventing unpleasant or harmful side effects, since such drugs would not affect the pool of uncomplexed SFKs involved in other cellular activities.

The data hereby summarized lead us to assume that, despite the widespread occurrence of PRMs on VPs and the existence of over 200 SH3 domains, PRMs are directed to specific host targets, among which SFKs are crucial actors in sustaining virus survival. Besides, the interaction between SFKs and PRMs of VPs seems to be emerging as a novel issue of special interest in the light of their association with virulence of viral strains and the level of pathogenicity, as reported for AIV NS1 and HIV-1 Nef, with the enhancement of the kinase activity being a sort of epiphenomenon related to the severity of disease. In this respect, retrospective studies on highly pathogenic virosis and structural analysis of PRM-bearing proteins as well as their effect on SFKs would open new perspectives and provide further hints for pharmaceutical research and clinical applications.

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