How pathogens exploit interactions mediated by SH3 domains

An important event in signal transduction is the binding of SH3 domains, found in viral proteins such as v-Src and v-Crk, to proline-rich sequences. Recently, proline-rich proteins that bind host-cell SH3 domains have been identified in a variety of pathogenic microorganisms. Exploitation of SH3-mediated interactions by pathogens may be more common than previously suspected.

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The amino-terminal, non-catalytic region of the v-Src kinase contains two modular domains, known as Src homology region 2 (SH2) and Src homology region 3 (SH3), that are conserved in a large number of signal transduction proteins [1,2]. SH2 domains are ~ 100 amino acids in length and bind to proteins that are phosphorylated on tyrosine within a specific consensus motif. SH3 domains are ~ 60 amino acids in length and bind to sequences that adopt a left-handed polyproline II helical structure. Most SH3-binding sequences contain at least two prolines and conform to the general consensus motif PxxP, where P is proline and x is a non-conserved residue. SH2 and SH3 domains can be thought of as modular cassettes that are inserted into proteins in different locations and in different combinations to promote highly specific protein–protein interactions. These domains are found in structural proteins, in enzymes (e.g., v-Src) and in proteins with no other apparent functional domains. This latter class of SH2/SH3-containing proteins function as 'adapters' or 'molecular adhesives' that participate in the formation of multiprotein complexes [3].

Given the importance of SH2 and SH3 domains in normal cellular physiology, it is not surprising that pathogenic microbes, both viruses and bacteria, have acquired these protein modules from their eukaryotic hosts and adapted them for their own uses. One of the more intensively studied viral SH2/SH3-containing proteins is v-Crk, originally identified as the oncogenic agent of the avian sarcoma virus CT10 [3]. v-Crk mediates CT10-induced tumor formation in chickens, and in cultured cells v-Crk induces cellular transformation associated with alterations in cell morphology, increased adhesion-independent growth, and enhanced tyrosine phosphorylation of several proteins. The name Crk, for CT10 regulator of kinase, is derived from this latter property. Cellular transformation mediated by v-Crk probably provides a selective advantage to CT10 by enhancing the rate of viral replication.

More recently, viral proteins that interact with host cell SH3 domains via PxxP motifs have been identified. One such protein is Nef of HIV-1 [4]. Interaction of Nef with host cell SH3 domains seems to enhance HIV-1 infectivity through modulation of host cell signaling functions. These findings suggest that microbial exploitation of SH3-mediated interactions may be a widely used infection strategy. In the following sections, I will briefly review the molecular basis of the interaction of SH3 domains with PxxP motifs and then discuss the role of these interactions in microbial pathogenesis, focusing primarily on v-Crk and recently described viral SH3-binding proteins.

Molecular basis of SH3 domain–PxxP interactions

Analysis of the three-dimensional structure of several different SH3 domains has revealed a common organization consisting of two anti-parallel β sheets packed against each other to form a shallow peptide-binding surface [2,5]. The binding surface is dominated by a patch of conserved hydrophobic residues, which is flanked on one side by a cluster of charged amino acids formed by the variable RT loop (so called because it contains arginine and threonine residues that are critical to the function of Src) and the variable n-Src loop (which contains an insertion in the sequence of neuronal Src). The helical structure of the bound ligand resembles a trigonal prism, with two edges of the prism directly contacting the surface of the SH3 domain (Fig. 1). The two conserved prolines in the PxxP motif form extensive interactions with hydrophobic residues in the SH3 domain. Two different classes of PxxP motifs have been identified, depending on the orientation in which they bind SH3 domains [2,6]. Class I motifs bind in the 'plus' orientation and generally contain charged residues on the amino-terminal side of the PxxP motif. Conversely, Class II motifs contain charged residues carboxy-terminal to the PxxP motif and bind in the opposite, or 'minus', orientation. Binding orientation seems to be determined by electrostatic interactions between the basic residues that flank the PxxP motif and the acidic residues in the RT loop of the SH3 domain [2,6].

Proline-rich peptides generally bind to SH3 domains with dissociation constants (K_d values) in the low micromolar (μM) range, as determined by fluorescence spectroscopy. Binding orientation does not seem to correlate with binding affinity, as high and low affinity peptides that bind in either orientation have been identified. Certain peptides

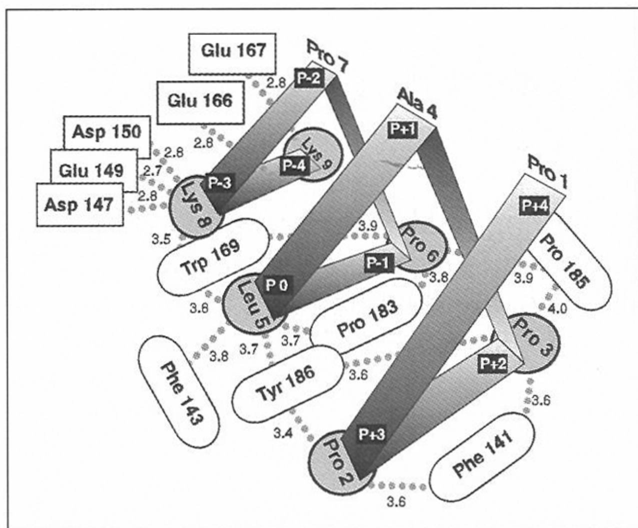

Fig. 1. Schematic diagram showing interactions between a proline-rich peptide from C3G and the c-Crk SH3-N domain. The C3G peptide is represented by the left-handed ribbon, and peptide residues that interact with the SH3 domain are indicated by the shaded circles. Residues in c-Crk SH3-N that interact with the peptide are indicated as oval boxes (for hydrophobic residues) and rectangular boxes (for acidic residues). Conserved prolines in the PxxP motif correspond to Pro3 and Pro6. Distances (in Å) between interacting residues are shown for the nearest pair of carbon atoms (for hydrophobic interactions) and between donor atom and acceptor atom for hydrogen bonds. Reprinted with permission from [13].

bind with high affinity to several different SH3 domains, whereas others are more specific. As discussed below, it seems that subtle differences in ionic contacts between peptides and SH3 domains are critical determinants of binding affinity and specificity.

The v-Crk SH3 domain in CT10-induced cellular transformation

v-Crk is a 47-kDa protein consisting of a viral-derived Gag (coat protein) sequence followed by a single SH2 domain and a single SH3 domain [3] (Fig. 2). Three cellular homologs of v-Crk have been identified: c-Crk I and c-Crk II, which arise from alternatively spliced transcripts, and a third distinct protein called Crk-L (for Crk-like protein) (Fig. 2). Whereas v-Crk and c-Crk I share a similar organization, c-Crk II and c-Crk-L contain a carboxy-terminal spacer region followed by an additional SH3 domain. Recent evidence suggests that the cellular Crk proteins are involved in signal transduction pathways dependent on Ras, or Ras-related GTPases [3]. Two proteins that are highly phosphorylated in CT10-transformed cells and that bind the Crk SH2 domain are Cas (Crk-associated substrate) and paxillin [3]. Cas and paxillin seem to be involved in signaling between extracellular matrix receptors and the cytoskeleton [3,7], but a direct link between these proteins and Ras-dependent signaling pathways remains to be shown.

The SH3 domain of v-Crk binds *in vitro* to multiple PxxP-containing proteins including the tyrosine kinases Abl and Arg, the guanine nucleotide exchange factors (GNEFs) Sos and C3G, and the epidermal growth factor receptor substrate Eps15 [3,8]. v-Crk has been shown to bind Abl and C3G constitutively *in vivo*. In contrast, binding of Abl to the amino-terminal SH3 domain (SH3-N) of c-Crk II (hereafter referred to as c-Crk) seems to be transient, and can only be detected *in vitro*, or when the proteins are highly overexpressed *in vivo* [3,9]. Abl phosphorylates a tyrosine (Tyr221) in the spacer region between the SH3 domains of c-Crk under conditions that favor their interaction [3]. This SH3-mediated selection of c-Crk as a substrate by Abl may be important in negative regulation of c-Crk. Intramolecular binding of the c-Crk SH2 domain to phosphorylated Tyr221 folds the protein into an inactive conformation, sequestering both the SH2 domain and the first SH3 domain (SH3-N) from interactions with other proteins [3,9,10]. v-Crk lacks this regulatory site of phosphorylation and thus may be constitutively active.

Mutational analysis of CT10 has shown that the SH2 and SH3 domains of v-Crk are critical for transforming activity [11,12]. For example, either deletion of the SH3 domain, or insertion of a linker mutation into the RT loop was associated with a significant reduction or a complete loss in

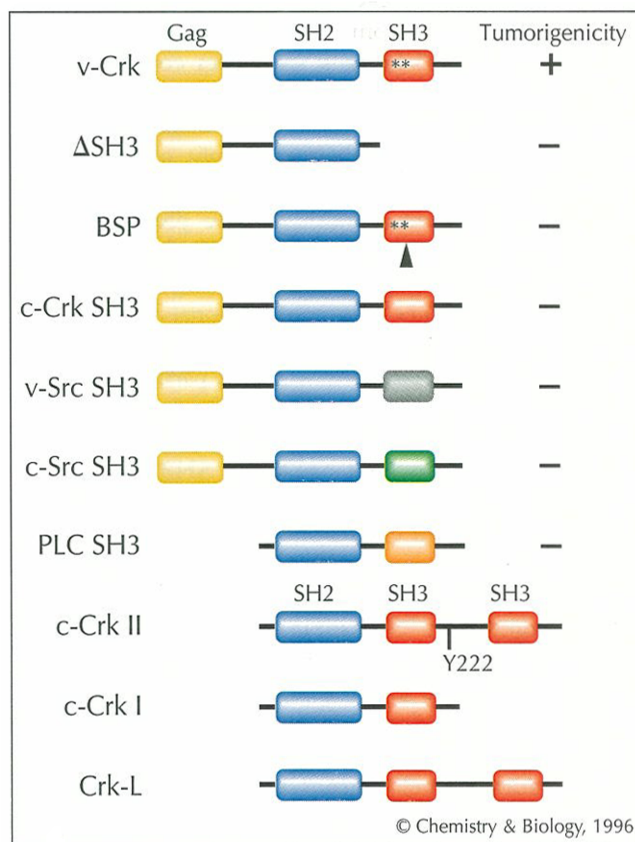

Fig. 2. Structures of wild-type and mutant forms of v-Crk and their relationship to cellular Crk proteins. Schematic representations of the domains (Gag (viral coat protein), SH2 and SH3) in various Crk proteins are shown. The ability of wild-type v-Crk or various deletion (ΔSH3), linker insertion (BSP) or chimeric v-Crk proteins to mediate CT10-induced tumor formation in chickens is indicated. Asterisks indicate the positions of amino-acid substitutions (Asn141→Lys and Glu149→Gly) in the v-Crk SH3 domain. Arrowhead indicates the position of the linker insertion in the BSP mutant. In c-Crk II, the tyrosine (Tyr221) phosphorylated by Abl is shown.

viral tumorigenicity (Fig. 2). Replacement of the v-Crk SH3 domain with SH3 domains from PLC-γ, v-Src or c-Src also resulted in a complete loss in tumorigenicity (Fig. 2). Surprisingly, a chimera of v-Crk containing the SH3-N domain from c-Crk has also been reported to be non-tumorigenic [11]. One explanation for this finding may be the fact that the sequence of the v-Crk SH3 domain differs from that of the c-Crk SH3-N domain at two positions. These substitutions lie within the RT loop and correspond to conversion of Asn141 to lysine and Glu149 to glycine.

Analysis of the three-dimensional structure of proline-rich peptides bound to the c-Crk SH3-N domain has revealed that the binding specificity of the Crk SH3-N domain is determined by the nature of the ionic contacts formed between acidic residues in the RT loop, in particular Glu149, and the first basic residue following the PxxP motif [13]. Glu149 forms a highly specific interaction with the lysine that follows the PxxP motif in a peptide derived from C3G, whereas a peptide derived from Sos, which contains an arginine after the PxxP motif, does not form this interaction. This may explain why the C3G peptide binds selectively and with high affinity to the c-Crk SH3-N domain, whereas the Sos peptide binds with somewhat lower but equal affinities to the Crk and Grb2 SH3-N domains (Table 1). Because the v-Crk SH3 domain contains a glycine at position 149 instead of glutamic acid, it binds the C3G peptide with lower affinity and is not sensitive to the presence of lysine versus arginine in peptides (Table 1). The loss in SH3-binding selectivity resulting from this mutation may contribute to the oncogenic potential of v-Crk. A model for v-Crk-induced tumorigenicity which incorporates these concepts is shown in Figure 3.

Table 1. Binding affinities of proline-rich peptides for the SH3 domains of c-Crk, Grb2 (amino-terminal domains) and v-Crk.

Peptide	c-Crk SH3-N K_d (μm)	v-Crk K_d (μm)	Grb2 SH3-N K_d (μm)
PP**P**AL**P**PKKR (C3G)	1.89 ± 0.06	25.5 ± 0.2	142 ± 3
P**P**PV**P**PRRRR (Sos)	5.24 ± 0.16	23.7 ± 0.6	3.54 ± 0.16

Data are from Knudsen *et al.* [23]. The data shown are for glutathione S-transferase fusion proteins and were obtained by measuring the changes in intrinsic tryptophan fluorescence upon peptide binding. Conserved prolines in the PxxP motif are indicated in bold.

Interaction of viral PxxP-containing proteins with host cell SH3 domains

The Nef protein

Nef is a 27-kDa protein encoded by human (HIV-1 and HIV-2) and simian (SIV) immunodeficiency viruses [14,15]. Although the exact role of Nef in the viral life cycle is controversial, Nef function is required for production of high viral loads of SIV and for progression of acquired immune deficiency syndrome (AIDS) in a Rhesus-monkey infection model [14,15]. More recently, an epidemiological study has documented a lack of AIDS progression in a small group of humans infected with Nef-defective HIV strains [16]. Functional studies of Nef suggests that it reduces the expression of the CD4 receptor on the T-cell surface and enhances the ability of HIV particles to establish productive infections of primary (i.e., non-immortalized) cells, such as peripheral blood mononuclear cells [14,15]. CD4 is the high-affinity receptor used by HIV to attach to and enter cells. Downregulation of CD4

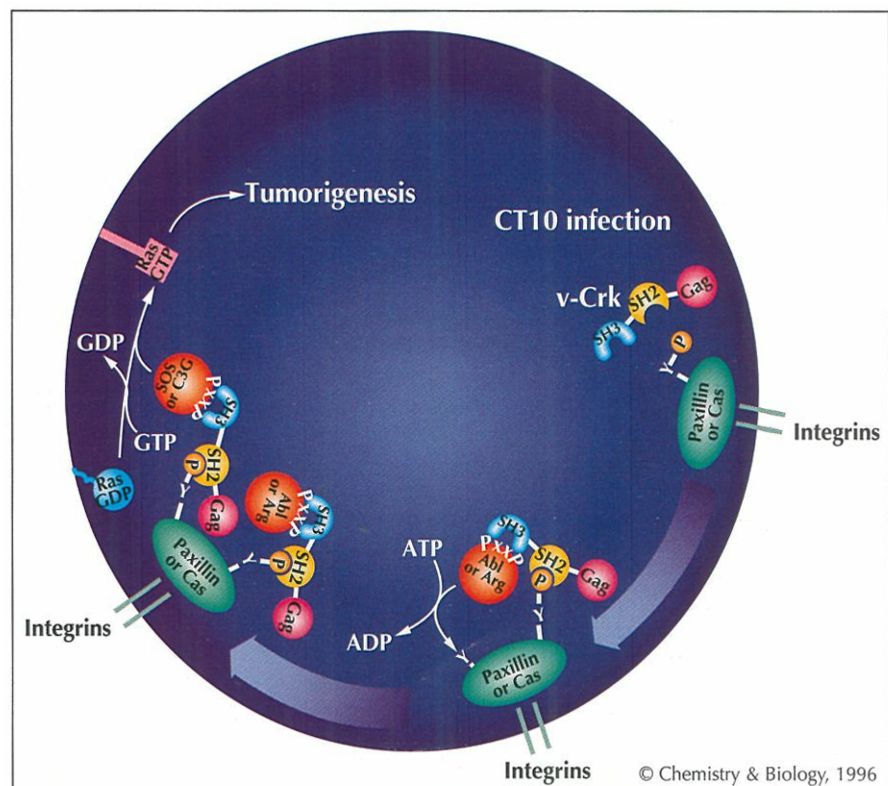

Fig. 3. A model for v-Crk-induced cellular transformation. v-Crk expressed after viral infection binds via SH2 to Cas and paxillin molecules that are constitutively tyrosine phosphorylated at a low level in the host cell. Tyrosine kinases (Abl and/or Arg) are then recruited to these sites by the v-Crk SH3 domain. Hyperphosphorylation of Cas and paxillin results from the processive action of Abl and/or Arg, leading to the formation of additional v-Crk binding sites. Guanine nucleotide exchange factors (GNEFs; e.g., Sos and C3G) are then recruited to these sites by v-Crk SH3 domains. The apparent non-selectivity of the v-Crk SH3 domain may contribute to the recruitment of inappropriate levels or types of GNEFs. The focal accumulation of GNEF activity at the membrane leads to the constitutive activation of a Ras or Ras-related signaling pathway.

is expected to prevent additional cycles of infection (superinfection), a common survival strategy used by many viruses. To enhance viral infectivity, it seems either that Nef acts within cells that are producing virion particles (producer cells), or that it is carried along with the virion to act in the target cell, since *de novo* synthesis of Nef is not required within target cells [15].

The central region of Nef contains a conserved proline rich sequence with three potential PxxP motifs [4] (Fig. 4). Mutational analysis has indicated that the PxxP motifs are dispensable for downregulation of CD4, but one or more of them are required for enhancement of viral infectivity [4,17]. The mutant viruses used in these studies encoded Nef proteins in which all three conserved PxxP motifs were eliminated by alanine substitutions, so it was not possible to identify a single PxxP motif that was critical for this function. Saksela *et al.* [4] also showed that, *in vitro*, wild-type Nef bound to SH3 domains from the Src-related kinases Hck and Lyn, but not Fyn or Lck, and that this binding activity was dependent upon intact PxxP motifs. The interaction of Nef with the Lyn SH3 domain was sensitive to substitutions made at a position adjacent to one of the PxxP motifs, a position which varies between laboratory and patient-derived strains of HIV-1. Interaction between Nef and Hck or Lyn *in vivo* has not been demonstrated and may be transient, or an SH3-containing host protein(s) that has not yet been identified may bind Nef with higher affinity. Other domains of Nef were also shown to be important for enhancement of viral infectivity [17], so Nef probably interacts with several types of host molecules in a cooperative manner. In this regard, it has been shown that a host-cell protein with serine kinase activity co-precipitates with Nef [14].

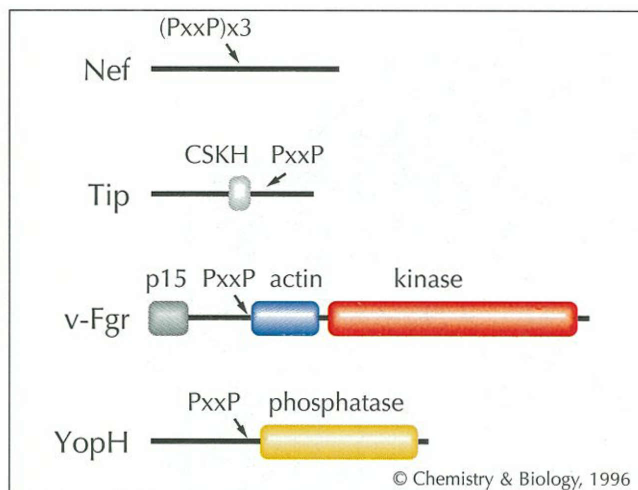

Fig. 4. Structures of microbial PxxP-containing proteins. Schematic representations of Nef, Tip, v-Fgr and YopH are shown. Locations of PxxP motifs are indicated by arrows. Other functional domains or regions of homology with other viral or cellular proteins are indicated by boxes. In Tip, CSKH indicates carboxy-terminal Src kinase homology region. In v-Fgr, p15 is derived from the viral p15 protein, actin corresponds to homology with cellular actin and kinase represents the tyrosine kinase domain. In YopH, the tyrosine phosphatase catalytic domain is indicated.

The herpesvirus Tip protein

Tip is a 40-kDa protein encoded by the first open reading frame of herpesvirus saimiri (HVS). This protein seems to be essential for HVS-mediated transformation of T cells from humans and New World primate species [18,19]. Analysis of HVS-transformed human T cells reveals that Tip is associated with the Src-related tyrosine kinase Lck [19]. Moreover, when kinase reactions were performed on immunoprecipitated complexes of Tip and Lck, Tip became phosphorylated on tyrosine, suggesting that the viral protein is recognized as a substrate by the kinase.

The 256-amino-acid Tip protein contains two regions related to host-cell signal transduction proteins: a short region related to a regulatory domain in Src kinases (the carboxy-terminal Src kinase homology region), and a PxxP motif related to a Src SH3-binding consensus motif (Fig. 4). Both regions were shown to be essential for interaction of Tip with full length Lck *in vitro* and in transfected cell lines that overexpressed both proteins [20]. The two regions seem to cooperate to enhance the affinity of Tip binding to Lck, although it will be important to confirm these findings in HVS-transformed T cells and to demonstrate a role for the Lck SH3 domain in this interaction. Lck has been shown to bind a number of different signal transduction proteins in T cells, including the CD4 receptor. Interaction of Tip with Lck may modify the interaction of the kinase with the receptor, or other signal transduction proteins, leading to cellular immortalization and enhancement of viral replication.

A family of PxxP-containing microbial proteins

The development of consensus motifs for SH3 binding sites has facilitated the analysis of known SH3-binding proteins, and provided a tool to search for new SH3-binding proteins based on primary sequence alone [21,22]. For example, since it is known that Nef binds at least two Src-related SH3 domains, it is possible to align the proline-rich region of Nef with a Src SH3-binding consensus motif (class II) based solely on the positions of conserved proline and arginine residues (Table 2). A similar alignment can be achieved with the proline-rich region of Tip, which presumably binds the SH3 domain of the Src-related kinase Lck (Table 2).

Table 2. Alignment of SH3-binding motifs in microbial proteins.

SH3-binding motif	Sequence
Src SH3 Class I consensus[a]	RXL**P**PL**P**
v-Fgr 14–20	RPL**P**PL**P**
Src SH3 Class II consensus	X**P**PL**P**XR
Nef 71–77	T**P**QV**P**LR
Tip 176–182	T**P**PL**P**PR
YopH 158–164	T**P**PL**P**PR

[a]Consensus motifs are from Feng *et al.* [6]. Critical prolines are shown in bold. SH3-binding motif in v-Fgr and its alignment to Class I consensus is from Yu *et al.* [22]. SH3-binding motifs in Nef [4], Tip [20] and YopH (D. Black, personal communication) were aligned by eye using conserved proline and arginine residues.

Although this does not prove that Nef or Tip bind SH3 domains in a class II orientation, it does provide a rational basis for mutational analysis of these SH3-binding proteins by single amino acid substitution. A comparative search of protein data bases with a consensus binding motif for the SH3 domain of phosphatidylinositol 3-kinase identified a potential SH3-binding site in the viral tyrosine kinase v-Fgr [22] (Table 2). Work in our own laboratory has characterized an SH3 binding site in a bacterial tyrosine phosphatase, YopH, that is produced by pathogenic *Yersinia* species (D. Black *et al.*, unpublished data) (Table 2). These results suggest the existence of a family of microbial proteins that interact with SH3 domains via PxxP motifs. In the case of v-Fgr and the *Yersinia* tyrosine phosphatase, interaction with host cell SH3 domains could serve to localize these enzymes within cells, to regulate their activities, or to recruit substrates to their catalytic domains.

Conclusions

Significant advances have recently been made in our understanding of SH3 domains and their roles in signal transduction. It is now clear that SH3 domains mediate protein–protein interactions by binding to polyproline II helices that conform to the general consensus PxxP. The rules that govern the selectivity and affinity of these interactions are being deciphered at the molecular and atomic levels. The modular nature of SH3 domains and PxxP-containing sequences allows for their insertion into proteins in many different positions and combinations. When combined with other types of protein domains, such as SH2, tyrosine kinase or tyrosine phosphatase domains, SH3 domains and PxxP motifs allow for the formation of highly specific, multiprotein signaling complexes.

The exploitation of SH3-mediated interactions by pathogenic microorganisms follows a common theme in host-parasite interactions; microorganisms that adapt key elements of the host cell for their own devices gain a selective advantage. In the examples cited here, interactions mediated by SH3 domains seem to allow for the modulation of host cell signaling pathways in ways that enhance microbial replication and/or infectivity. The variation in SH3 selectivity by mutation that is apparent in two of the examples cited here (v-Crk and Nef) may be important in this regard. As an extension of these findings, it may be possible to design therapeutic reagents that treat infectious diseases by interfering with SH3-mediated interactions. By targeting the cellular counterpart of the microbial protein, it may be possible to tip the balance in favor of the host while problems associated with mutational variation on the part of the microbe would be avoided.

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