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REVIEW

Peptide-Based Inhibitors of the Induced Signaling Protein Interactions: Current State and Prospects

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Abstract—Formation of the transient protein complexes in response to activation of cellular receptors is a common mechanism by which cells respond to external stimuli. This article presents the concept of blocking interactions of signaling proteins by the peptide inhibitors, and describes the progress achieved to date in the development of signaling inhibitors that act by blocking the signal-dependent protein interactions.

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INTRODUCTION

Formation of multicomponent protein complexes of a definite structure in response to activation of cellular receptors is a common mechanism by which cells translate external signals into a biologically relevant response. The general principles of operation of such complexes, often called signaling complexes, were described at the end of the last century [1, 2]. This conceptual understanding was complemented in the recent two decades with achievements in structural biology revealing the fine structure of many oligomeric complexes consisting of signaling proteins or their separate domains with resolution sufficient for visualizing localization of individual atoms [3]. This knowledge significantly expanded our understanding of the mechanisms of assembly of such complexes and their functioning. At the same time, it has been recognized that the data accumulated so far are fragmentary and do not reflect dynamics of real complexes. Furthermore, considering that majority of the complexes have been resolved using recombinant DNA technology, there is a problem in validation of physiologically relevant structures.

Formation of signaling complexes in the vast majority of cases is mediated by specialized protein domains often called protein interaction domains (PIDs) [2, 4]. Some of the PIDs, such as SH2, SH3, or bromodomains recognize specific features or short motifs present in the protein primary structure, while other domains such as TIR-domains [homology domain between insect Toll-protein, human IL-1R (interleukin 1 receptor), and plant disease-resistance proteins] or death domains (DDs) do not have specific binding motifs. PIDs of the last type can establish multiple cooperative interactions with the domains of same type either homo- or heterotypically [5, 6]. Currently available structures of signaling complexes demonstrate wide topological diversity and uniqueness of the sites mediating individual binary interactions of the components within the oligomeric complexes [3]. Structural diversity of signaling complexes includes

Abbreviations: ACE2, angiotensin-converting enzyme 2; AMP, antimicrobial peptide; BP, blocking peptide; BB-loop, the loop between second β-strand and second α-helix; CPP, cell-permeating peptide; DD, death domain; IL, interleukin; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response protein 88; PID, protein interaction domain; TIR, domain homologous in insect Toll proteins, human IL-1R, and plant disease-resistance protein; TIRAP, TIR-containing adapter protein, also known as Mal; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAM, TRIF-related adapter molecule, also known as TICAM-2; TRIF, TIR domain-containing adapter inducing IFN-β, also known as TICAM-1.

linear double-stranded complexes formed by TIR domains of activated Toll-like receptors (TLRs) and their adapter proteins, and by members of the IL-1R family [7, 8], as well as helical complexes of different structures, including single- and double-stranded helices, as represented by Myddosome [9], PIDDosome [10], MDA5-MAVS [11], inflammasomes [12-14], and others [3, 15, 16].

Cell signaling is an important pharmaceutical target. This notion is supported by more than 25 years of clinical success of therapeutic antibodies designed to block cellular receptors, as well as cytokines that activate these receptors [17-19]. Another indication of the importance of signaling pathways as pharmacological targets is the fact that small molecules that block binding of the G-protein-coupled receptors with ligands represent a significant part of the existing drug pool [20-23]. Considering that small molecules are not very effective in blocking receptors activated by macromolecules (such as cytokines or exogenous molecules from pathogens), as well as the fact that many signaling pathways do not have circulating agonists, it seems reasonable to assume that blocking of signaling pathways by disrupting assembly of signaling complexes could be a promising approach for the development of new pharmaceuticals. However, progress in the development of new pharmaceuticals with such mechanism of action is limited at present. At the same time, a large amount of experimental data obtained both *in vitro* using cultivated cells and in various *in vivo* models indicates that targeted development of inhibitors of signal transduction from the activated receptors by blocking assembly of signaling complexes is possible. Considering that the assembly of signaling complexes in the majority of cases is mediated through interactions between the PIDs [2], PIDs are among most popular targets of the inhibitors developed to date. This article reviews current progress in the development of signaling complex assembly inhibitors and discusses the prospects and further progress in the field.

CONCEPT OF PEPTIDE-BASED INHIBITORS OF THE TRANSIENT PROTEIN INTERACTIONS

Many examples (some of which will be discussed below) demonstrate that relatively short peptides corresponding to fragments of protein primary sequence comprising functional protein–protein interaction sites are capable of blocking protein function. Such peptides, often called blocking peptides (BPs) or decoy peptides, act through binding to the interaction sites of their prototype protein with its protein partners, thereby preventing formation of a functional complex and, subsequently, inhibiting downstream functions

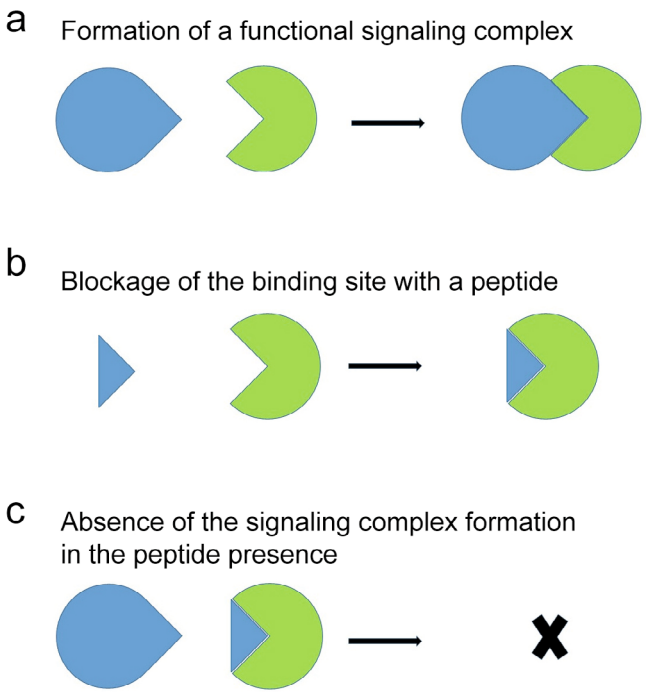

Fig. 1. Mechanisms of action of blocking peptides – prevention of formation of the protein complex by competing with the protein prototype for the functional binding site. a) Interaction of PID1 (blue shape) with PID2 (green sector) results in formation of a functional binary complex. b) Peptide corresponding to the PID1 binding site of PID2 (blue triangle) retains its ability to bind PID2. c) Binding of the peptide with PID2 prevents formation of the PID1–PID2 complex.

(Fig. 1). Because the assembly of signaling complexes is an intracellular process, the segment mimicking the interaction site must be complemented by the segment facilitating permeation of the construct into the cell. Peptide vectors, which are either derived from a natural protein or artificially obtained, are most often used for facilitation of transmembrane transfer of blocking peptides. Hence, the peptide-based blockers of interactions between cytoplasmic proteins consist from two functional parts: a specific part designed to mediate binding with the target proteins and a peptide vector required for intracellular permeation of the construct. Below typical features of these two parts will be discussed separately.

Target-binding segment of blocking peptides. Functional role of this peptide part is to block the prototype protein interactions, causing the consequent prevention of signaling complex formation (Fig. 1). In the cases when the site of interaction in the protein is known, design of blocking peptides seems obvious. However, results of screening of large peptide libraries have demonstrated that a large fraction of the peptides, which do correspond to the binding site, nevertheless do not exhibit inhibitory activity in *in vitro* or *in vivo* tests [24-26]. The most often factors accounting for the lack of functional activity in these cases

are nonspecific peptide binding by the components of cell proteome, insufficient peptide solubility, negative effect of the target-binding part on the peptide permeability, biological instability of the peptide, and others [27]. In the cases, when boundaries of the binding site are unknown, inhibitory sequences are determined via screening of the peptide libraries or selected empirically [5, 26, 28]. Typical size of binding sites involved in the transient interactions of signaling proteins should be taken into consideration in the case of empirical selection. General characteristics of protein interaction sites have been investigated in detail and described in a number of publications [29-32]. Analysis of the known structures of signaling complexes formed by PIDs confirms these general characteristics and further specifies that the typical surface area of binary interactions in signaling complexes (250-800 Å^2) is lower than the average size of protein interactions in general [7-9, 33]. Taking into account that circularity of the interaction sites in globular proteins typically is high (0.7, as an average [29]), upper estimate of a binary interaction site in such complex could be modelled by a circle of ~30 Å diameter. Alpha- or 3_{10}-helices of such length would correspond to the peptides consisting of 20 or 15 aa, respectively, while the peptide of same length in the β-strand conformation would consist of 8-9 aa [34]. These simplified estimates correlate quite well with the range of sizes of target-binding segments in the experimentally verified blocking peptides [26, 27].

Vectors facilitating intracellular penetration of the blocking peptides. Peptide-based vectors are frequently used to carry macromolecules across plasma membrane. In the late 1980s, a natural capability of Transcriptional transactivator (Tat) protein of Human immunodeficiency virus to translocate across the plasma membrane was discovered [35, 36]. Soon after this discovery, a similar capability to translocate across plasma membranes was discovered for the Drosophila Antennapedia homeodomain [37]. It was shown that the permeating capability of Antennapedia homeodomain is due to the presence of the 16 aa-long segments of the primary sequence, which forms the third α-helix of the protein [38]. Later, functionally similar short sequences (often called cell-permeating or cell-penetrating peptides, CPPs) have been found in Tat and other proteins capable of permeating cell plasma membranes [39-42]. Today, more than 1000 CPPs of different types have been discovered [43, 44]. In addition to CPPs from natural proteins, artificially created CPPs have been reported [40, 42, 45]. Based on physicochemical properties of peptides, the following types of CPPs are recognized: cationic, hydrophobic, and amphipathic consisting of cationic and hydrophobic parts [45]. Permeating sequences of Tat (RKKRRQRRR) and Antp (RQIKIWFQNRRMKWKK) proteins, as well as other members of the family of transcription factors that contain homeodomain, are examples of cationic CPPs [42, 46]. Several artificial sequences, e.g., oligoarginines or oligolysines, also belong to this group [45, 47]. Transportan, an artificially created combination of fragments of two natural proteins, is an example of amphipathic CPP [48]. There are only a few known hydrophobic CPPs, they are less sensitive to amino acid substitutions [45]. Lower efficiency of hydrophobic CPPs in comparison with the CPPs of other types has been noted.

CPPs internalization mechanisms remain a subject of discussion in the literature [42, 49]. It is recognized that CPPs are internalized via either endocytosis with subsequent release from endosomes, or via direct interaction with the plasma membrane; moreover, individual CPPs could use multiple mechanism for penetration with their importance differing for individual peptides and cell lines. At the same time, CPPs do not use protein transporters for cell penetration, which explains versatility of their action with respect to different cell types. Length of the used CPPs varies in the range 5-30 aa. In addition to proteins and peptides, CPPs are capable of transporting macromolecules of different nature to the cells including nucleic acids, medicinal preparations, and contrast agents, as well as complex supramolecular structures such as liposomes and nanoparticles [45, 49, 50]. Peptide vectors are efficient for intracellular transport of various compounds not only in a monolayer of cultivated cells. Numerous studies demonstrated that CPPs also increase tissue permeability, including permeability of blood-brain barrier. In particular, it was shown in the early study by Schwarze et al. [51] that Tat-peptides facilitated penetration of the intraperitoneally administered β-galactosidase, a 120-kDa protein, into all tissues including brain. Subsequent studies confirmed efficiency of CPPs for intracellular transport of cargo of different nature *in vivo*, as well wide distribution of cargo in the organs, which was shown in the cases of both intraperitoneal and intravenous injections [52-55]. At the same time, predominant accumulation of CPPs in liver and kidneys was noted, while accumulation in brain and muscles was significantly lower [53, 54].

It is worth mentioning that typical working concentration of CPPs in cell culture are in a narrow range varying from 1-5 μM to 50-100 μM for a wide variety of CPPs, transported agents, and cell lines [26]. The most plausible reason for existence of the lower limit of effective concentration is low efficiency of transport at lower concentrations of CPPs. This is confirmed by significantly higher affinities of blocking peptides to their protein targets demonstrated *in vitro* using a recombinant protein, in comparison with the effective peptide concentration in cell culture. One of the examples is the TLR-blocking peptide, 2R9, which exhibits

a ~40 nM binding affinity to its target, TIR-domain of the adaptor protein TIRAP, while the effective concentration of 2R9 in macrophage cell culture is almost 500-fold higher [56]. Existence of the upper limit of CPP concentration used in cell is due to cytotoxicity often observed, when concentration of BP is above 100 μM, which is due to their membranotropic action and ability to form membrane pores at high concentrations.

Another manifestation of CPP membranotropic activity is their antimicrobial properties, which are due to similarity of their physicochemical properties with the properties of antimicrobial peptides (AMPs) [57, 58]. AMPs are plant and animal peptides that function as an element of antimicrobial defence effective against various classes of pathogens, including bacteria, viruses, and fungi [59]. Both classes of the peptides, CPPs and AMPs, are predominantly represented by cationic peptides, which also contain a high number of hydrophobic amino acids [57, 60, 61]. Both CPPs and AMPs exhibit less cytotoxicity to animal cells in comparison with their activity against prokaryotes.

Despite the discovery of many new CPPs, two vectors discovered first, the Tat-peptide and the vector based on Antp (also known as penetratin), are most frequently used as CPPs. It must be mentioned that relative efficiency of these vector varies in different systems and depends on both the cell model and the nature of molecular target: in some systems Tat has been found to be more efficient, and in others – penetratin. There is no unambiguous and verifiable explanation for this fact in the literature, and primary selection of CPP is performed by the researchers empirically. One of the good features of penetratin is simplicity of quantification of the penetratin-containing BPs. Penetratin contains tryptophans, and, hence, its concentration can be determined spectrophotometrically, while existence of such option for the BPs containing Tat depends of the presence of absorbing amino acid residues in the blocking part of the peptide. It must be mentioned that natural sequences mediating penetration of a protein into the cell are evolutionary very well preserved. In particular, penetrating sequence of the Drosophila protein Antp is 100% preserved in the human homolog [62]. The high degree of evolutionary conservation of penetrating sequences reflects their biological significance for the function of proteins, which contain such sequences.

It is worth noting in conclusion of this section that the concept of blocking peptides is based on the often-observed ability of the peptides corresponding to the fragment of the primary sequence of the protein forming the site (or major part of the site) of the functional protein–protein interaction to bind the protein partner, and, as a result, to block this function of the prototype protein. The concept of BPs is applicable to intracellular targets if the peptide vectors capable of transporting the specific blocking part of the peptide inside the cell are used. The highest number of the currently known examples of successful use of BPs is related to the blocking of interactions mediated by PIDs, protein domains specialized in the protein–protein interactions associated with signaling.

FUNCTIONAL CHARACTERISTICS OF BLOCKING PEPTIDES

First successful attempts to modulate biological functions with synthetic BPs have been reported in 1990s. The first targets of these studies were extracellular protein interactions. In particular, different variants of the integrin-binding motif- (RGD) containing peptides were used as agents to block cell adhesion in attempts to develop new medicinal preparations [63]. Another early example of realization of the BP concept is Akt protein kinase inhibition by peptides-pseudosubstrates, as well as by the BP containing the βA strand of TCL1 protein [64, 65]. Use of peptide-based cell-permeating vectors in combination with the segments that provide the target binding specificity significantly expanded applications of the blocking peptide strategy. In particular, the peptides blocking NEMO (nuclear factor kappa-B (NF-κB) essential modulator) oligomerization, as well as peptides corresponding to the NEMO-binding domain of IKK, inhibited activation of NF-κB induced by tumor necrosis factor (TNF) or lipopolysaccharide (LPS) [66-68]. The peptide containing the 10 aa Tat sequence in combination with the 20 aa JNK-binding motif of JNK interacting protein 1 was used in the study by Borsello et al. [69]. Interestingly, both L- and D-isomers of this peptide blocked the kinase activity of JNK and exhibited neuroprotective effect in the brain ischemia models [69]. Another example of the protein segment blocking the mitogen-activated protein kinases (MAP) activity is the MEK1-derived sequence (13 aa), which effectively inhibited the activity of ERK kinases [70]. A peptide containing C-terminal segment of $G\alpha_s$ protein linked with penetratin is an example of successful realization of the BP concept with respect to G-protein-coupled receptors. This peptide specifically inhibited cAMP production stimulated by adrenoreceptor agonists [71].

A number of studies used the BP concept for development of TLR inhibitors. Horng et al. [72] were the first to use synthetic cell-penetrating peptides for inhibiting TLRs. The authors demonstrated that the peptide consisting of the BB-loop (loop between second β-strand and second α-helix of the TIR domain) of adapter protein TIRAP linked to penetratin blocked the LPS-induced activation of NF-κB and MAP-kinases in a macrophage cell line. This peptide, however, did not exhibit inhibitory activity when the cells were

stimulated with a TLR9 agonist or IL-1 [72]. The TIRAP peptide also selectively blocked production of the TLR4-dependent cytokines and dendritic cell maturation [72]. Another study compared the effects of BPs containing the BB-loop of 4 adapter proteins that mediate the TLR signal transduction [73]. This and other studies have demonstrated that the peptides containing the BB-loop of the TIR-containing adapter proteins MyD88 (Myeloid differentiation primary response protein 88), TRIF (TIR domain-containing adapter inducing IFN-β, also known as TICAM-1), and TRAM (TRIF-related adapter molecule, also known as TICAM-2 inhibited TLR4 signaling [41, 73-76]. The inhibitory efficiency of individual adapter peptides however differed, with the BP from TRAM been most potent [73]. A peptidomimetic imitating the central part of the TRAM BP exhibited a cardioprotective effect in the mouse model of myocardial infarction [77]. Authors interpreted this observation as an indication of a suppression of the TLR4-dependent inflammation by the TRAM BP [77]. In the following study, the same group confirmed that the dimeric peptidomimetic imitating the TRAM BB loop inhibited the LPS-induced transcription of *IFN-β* and *CXCL10* in a dose-dependent manner [78]. This agent, however, demonstrated only partial selectivity as it also inhibited IFN-β induced by TLR8 and MDA5/RIG-I agonists [78]. TLR2 and TLR4 peptides designed similarly to the adapter BB-peptides blocked the activity of their respective prototype receptor and exhibited some cross-reactivity, while the peptide containing homologous sequence from TLR1 or TLR6 (these TLRs do not induce signaling as homodimers, but only through heterodimerization with TLR2) did not exhibit inhibitory activity [79].

The next step in the development of the blocking peptide methodology was the screening of PID-derived peptide libraries. This approach was based on then emerging understanding of binding versatility of the PIDs, together with the lack of information on the exact positions of the PID interaction sites. The first comprehensive library representing the entire PID surface was the library from the TIR of TLR4 [5]. The library comprised 11 peptides, each of which included a segment of TLR4 TIR primary sequence as the blocking part. The segments were selected such that each represented a non-fragmented patch of the domain surface. Penetratin was used as a penetrating sequence. Results of this study validated the BP concept and indirectly confirmed the assumption of multiple binding sites present in TIR domains. Five peptides inhibited the LPS-induced activation of MAP kinases and transcription factors, and the expression of cytokine mRNA [5]. Inhibitory effect was exhibited by the peptides that corresponded to the site connecting the TIR-domain with the transmembrane portion of TLR, the AB and BB loops, as well as α-helices B and D (Fig. 2, a and b) [5]. A follow-up study performed a screening of the similarly designed peptide library from the adapter TIRAP [80]. The screening also identified 5 active peptides derived from the following structural elements of the TIR-domain: the AB loop, and the α-helices B, C, D, and E [80]. Interestingly, activity of the BB loop peptide used in this study, peptide TR4, was significantly lower than the activity of the previously used TIRAP BB-peptide, which differed from TR4 by one hydrophobic amino acid residue [72]. Peptide libraries from the TIR-domains of adapter proteins of the MyD88-independent pathway, TRAM and TRIF, were screened later [55, 81]. Two active peptides were identified in each of the libraries. In addition to the previously identified peptides derived from the BB loop of both adapter proteins [73], the inhibitory activity was observed for the peptide TM6 from the third helix in TRAM and peptide TF5 from the second helix in TRIF [55, 81]. Using deletion analysis, the authors identified TM6-ΔC and TF5-ΔC, which are fully active, truncated (9 aa) versions of the corresponding parent peptides. Subsequent studies that screened analogous peptide libraries from TIR-domains of TLR2, TLR2 co-receptors (TLR1 and TLR6), TLR9, TLR7, and TLR5 identified inhibitory sequences in each of the screened libraries [25, 26, 56, 82, 83]. Based on the analysis of positions of the segments that represent the active BPs, it was concluded that the active peptides originate from four topologically conserved TIR domain regions responsible for the assembly of signaling complexes by activated TLRs (Fig. 2) [26, 83-85]. Two sites (S1 and S4) are located on the opposite sides of the TIR-domain near the edge-forming strands of the β-sheet. In addition to the amino acid residues forming the strand B, the site S1 could include segments of the loops AB and/or BB, and, as is the case for TLR2, TLR4, and TLR7 TIR, the segment connecting TIR with the transmembrane helix [5, 24, 26]. The site S4 is represented by the peptides corresponding to the strand E, as well as the adjacent to the stand E α-helix E [24-26]. The sites S2 and S3 are formed by three helical regions adjacent to the convex side of the β-sheet i.e. helices B, C, and D. The helices B and/or C form the S2 site; the helix D – site S3 (Fig. 2c) [26, 83]. It was proposed that the primary TLR signaling complexes assemble through mutual interactions of site S1 with site S4, and of site S2 with site S3 (Fig. 2, d and e) [26, 83]. This assumption is also based on the results of structural analysis of oligomeric complexes formed spontaneously *in vitro* by the recombinant TIR-domains of the adaptor proteins TIRAP and MyD88 [7, 8].

In addition to the BP design strategy mimicking the eukaryotic protein interaction sites, other studies used the approach based on the ability of some bacterial and viral pathogens to block antimicrobial defences of higher animals by producing proteins capable

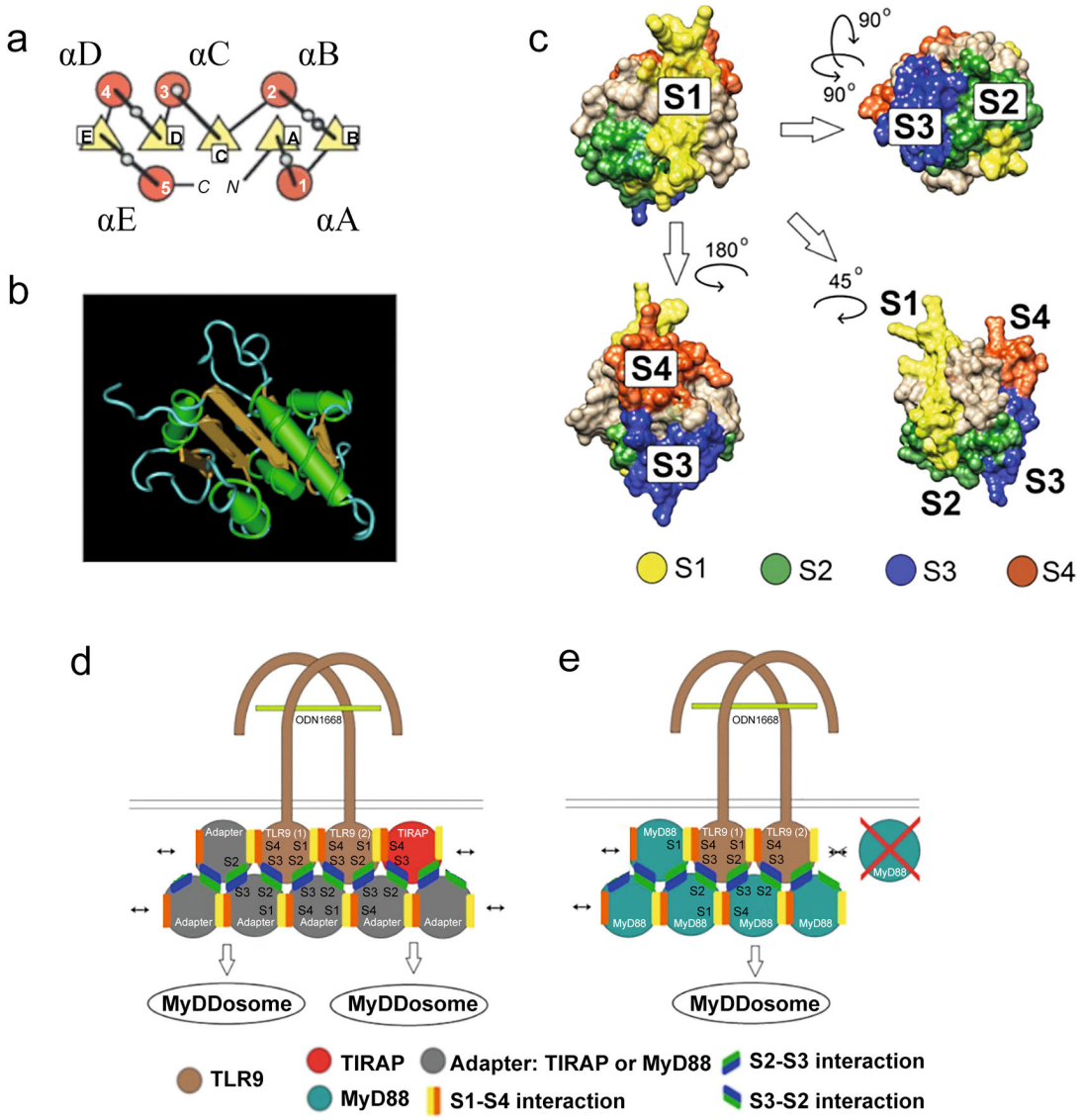

Fig. 2. The structure of TIR-domains, location of sites corresponding to the inhibitory BPs, and suggested architecture of TLR signaling complexes. a) Schematic representation of the secondary structure of TIR domains [84]. TIR domains belong to the class of α/β protein domains with the secondary structure consisting of alternating β-strands and α-helices [84]. According to the most popular nomenclature of the secondary structure elements TIR-domains are alphabetized starting from the N-terminus. For example, βA and βE [shown in panel (a) by triangles] denote first and fifth strands, and αB and αD – second and fourth helices (shown in the figure with brown circles). Loops are designated by two capital letters corresponding to the elements of the secondary structure they connect. For example, the BB loop connects the second strand with the second helix, and the DE-loop – fourth helix with fifth strand. b) Tertiary structure of the human TLR2 TIR domain [85]. A typical TIR domain consists of 5 strands arranged into a parallel β-sheet forming the domain core. α-Helices are located on both sides of the sheet – the first and the last helices are located at the convex side. c) Four binding sites of TIR domains that mediate receptor TIR dimerization and subsequent recruitment of adapter proteins are denoted as S1–S4. Sites S1 and S4 are located at the opposite sides of the TIR domain near the edge strands in β-sheet. Sites S2 and S3 are located in one semi-sphere. Site S2 is formed by helices B and/or C; site S3 – by helix D together with adjacent loops [26, 83]. d) TIR domain interactions in the signaling complex during TLR9 activation in the presence of both adapters of the MyD88-dependent pathway, TIRAP and MyD88. Sites S1 and S4 interact reciprocally and form links inside each strand of the two-stranded structure, while sites S2 and S3 form interchain connections [83]. e) Interaction of TIR domains in the signaling complex during TLR9 activation in the absence of TIRAP [83]. The complex can grow unidirectionally.

of binding the components of immune system [86, 87]. One example of such proteins is cowpox virus proteins A46R and A52R that have the ability to bind TIR domains of TLR4 and TLR4 adapter proteins, thereby blocking the assembly of TLR signaling complexes [88, 89]. These studies identified short peptides capable of inhibiting TLR in both viral proteins. In particular, the A52R-derived, polyarginine-linked peptide P13

effectively inhibited cytokine production induced by the TLR3, TLR4, and TLR7 agonists in cultured cells, and reduced the lethality in the mouse model of septic shock induced by administration of LPS and D-galactosamine [90, 91]. Screening of the A46R peptide library identified a potent TLR4 inhibitor effective in cell culture at 1–5 μM concentrations [28]. This peptide, named VIPER, was specific towards TLR4 and did not inhibit TLR2, TLR3, or TLR9. The authors systematically investigated the effects of amino acid substitutions on VIPER activity. Interestingly, substitution of a single amino acid at any position except the central leucine did not suppress the inhibitory activity of the peptide [28].

Bacterial TIR domain-containing virulence factors TcpB and TcpC are capable of inhibiting TLR signaling due to MyD88 bindings [92]. Snyder et al. [93] examined the BB and DD loop peptides from the TcpC TIR domain. Both peptides suppressed the LPS-induced macrophage activation. By using immunoprecipitation, the authors demonstrated that the BB loop peptide binds TLR4, whereas the DD peptide binds MyD88 [93]. Ke et al. [94] screened the peptide library of the TIR-domain from TcpB (TIR-containing protein in *Brucella*). The screening identified two peptides, TB-8 and TB-9, both of which inhibited response to LPS in *in vitro* and *in vivo* models [94].

Blocking peptides also were used in attempts to develop antiviral agents that block the entry of SARS-CoV-2 virus into host cells [95]. The study examined peptides derived from viral Spike protein and from the human Spike receptor, angiotensin-converting enzyme 2 (ACE2) [95]. Based on the structure of the Spike–ACE2 complex, Loi et al. [96] designed a series of short peptides originating from different Spike-ACE2 interaction sites and demonstrated that these peptides reduce the binding of virus to ACE2-expressing cells when used separately or (more efficiently) in combination.

Thus, literature presented in this section demonstrates that peptide-based blockers can target interactions of extracellular proteins as well as interactions of proteins located in the cytoplasm. In the latter case, the blocking part must be supplemented with a peptide vector to facilitate the BP transmembrane transfer. Receptor signaling function could be suppressed via blocking either receptor-receptor or adapter-adapter interactions, or a combination of these. The screening of peptide libraries created from PIDs is often more productive due to multiple protein interaction sites typically present in a PID. Another general observation that could be made based on the analysis of available publications is a relatively narrow range of effective BP concentrations for suppression of the intracellular targets. Effective BP concentration in a cell culture typically is in the range of 5-40 μM, which could be explained by the efficiency of transmembrane transport by peptide vectors.

VERIFICATION OF THE MECHANISM OF ACTION OF BLOCKING PEPTIDES

The blocking peptide concept is based on the assumption that BPs retain, to a large extend, the specificity and affinity of interactions mediated by the corresponding site of their full-size prototype protein. However, in practice, BPs are identified based not on the binding with their protein target in an *in vitro* system, but in functional tests using either screening or existing knowledge of the location of the binding site required for realization of the function that should be inhibited. Obviously, verification of the mechanism of action of BPs identified based on inhibition of the function should include confirmation of either direct binding of the BP with the target protein or/and blocking of interaction of the target with the full-size protein-prototype of the peptide. Currently there are data confirming this mechanism of action for the vast majority of BPs. In particular, it was shown in one of the earlier studies using immunoprecipitation technique that the peptide corresponding to the BB loop of the TIR-domain in MyD88 prevents dimerization of MyD88 [97]. Piao et al. [56] demonstrated later using dot-blotting technique that the peptide derived from the BB-loop in the TIR-domain of MyD88 binds TIR-domains of TIRAP and TLR9, in addition to MyD88. Another example of multi-specific binding is the peptide 7R11 corresponding to the fifth helix of the TIR-domain of TLR7 (site S4; Fig. 2c). 7R11, but not a control peptide, was shown to bind TIR-domains of both MyD88 and TIRAP; while the peptide 7R9 from the 4th helix of the TIR-domain from TLR7 (site S3; Fig. 2c) was shown to bind TIRAP, but not MyD88 or the control protein [24]. It was reported in the later studies that many BPs derived from the TIR-domains demonstrate multi-specific binding interacting with a specific subgroup of TIR-domain, which correlates with the properties of full-size PIDs [26]. These observations indicate that BPs could bind several targets, which, in turn, define functional properties of each particular peptide.

At the same time, certain peculiarities in the specificity of binding of BPs derived from PIDs have been noted. In particular, the BPs corresponding to the 4th helix of the TIR-domain from TLR interacted with the TIR-domains of adapter proteins, but not with the TIR-domains of the receptors [26]. For example, the peptide 4R9 (D helix of TLR4) did not bind TIR-domains of TLR4 and TLR2 [5], but interacted with the TIR-domain of the TIRAP adapter [81]. The peptide 2R9 (4th helical region (D helix) of the TIR-domain from TLR2) also predominately interacted with the TIR-domain of

TIRAP, but not with the TIR-domains from TLR1, TLR4, TLR6, or TLR9 [56]. Similarly, the BPs from the 4th helical region of the receptors TLR1 and TLR6 predominantly interacted with the TIR-domains of adaptor proteins, MyD88 and TIRAP, respectively, but not with the TIR-domain of TLR2, which is a co-receptor of the protein-prototypes of these BPs [82]. Another example of specific interactions of BPs from TIR-domains is binding exhibited by the peptides of the adapter protein TRIF belonging to the MyD88-independent signaling pathway [81]. The peptide TF4 interacted with the TIR-domain of the TLR4 receptor, and the TF5 peptide – with the TIR-domains of both TLR4 and TRAM, but none of the TRIF-peptides exhibited binding with the adapter proteins of the MyD88-dependent pathway, TIRAP and MyD88 [81].

One of approaches that could confirm that binding of the protein with the peptide is indeed involved in the inhibition of function of this protein, could be matching of the apparent binding constant for the protein–BP pair measured in a cellular system with the constant of inhibition of the protein function measured also in the cellular system. In order to obtain such confirmation a system based on the Forster resonance energy transfer was developed in our research group to examine quantitatively binding of the peptides with their protein targets directly in the cell. A panel of plasmids was designed that encoded hybrid proteins consisting of the TIR-domain conjugated with a fluorescent label. To evaluate peptide finding with the TIR-domains, the fluorescently labeled TIR-domains considered as possible peptide targets were ectopically expressed in the HeLa cells, and the cells were incubated in the presence of blocking peptide labeled with the fluorescent dye capable of quenching fluorescence of the label on the TIR-domain [5, 98]. Binding of the peptide with the TIR-domain was evaluated from the quenching of fluorescence of the protein label manifested as a reduction of its fluorescence lifetime. Numerous examples discussed in detail in the previously published literature review [26], as well as in two papers published later [24, 25] demonstrated practically complete coincidence of the effective concentrations required for blocking protein function by the cell-penetrating BPs and for quenching fluorescence of the labelled TIR-domains. The apparent binding constants measured in a cellular system for the inhibitory BPs with the TIR-targets are in the range 1-20 μM for all efficiently binding BP-TIR pairs known at present [26]. Such relatively narrow range of the effective concentration for a sufficiently large group of BPs identified now is, likely, a consequence of the commonality of the mechanism for BP penetration through the cell plasma membrane. This hypothesis was confirmed by the fact that BPs demonstrate significantly higher binding constant in the *in vitro* tests using recombinant protein targets in comparison with the binding in the cellular systems. For example, the peptide 2R9 binds to the recombinant TIR-domain of the adapter protein TIRAP in solution with K_D ~ 40 μM, which is significantly lower than the apparent dissociation constant for this pair in the cellular system [56]. Differences between the binding constants measured *in vitro* and in the cellular system could be in part explained also by nonspecific binding of the peptide with the extra- and intracellular proteins. It was shown, in particular, that 2R9 binds to the serum albumin with K_D ~ 1.5 μM [56]. The experiments conducted with the help of surface plasmon resonance technique confirmed that high affinity of the peptides to TIR-domain is due to the high rate of association and low rate of dissociation [56].

It could be stated in conclusion of the section that the experimental data accumulated until now confirm binding of the target proteins with the blocking peptides as a main mechanism of BP action. And, although, some BPs derived from PIDs, similar to their prototype proteins, demonstrate ability to bind several proteins representatives of the same class of proteins, many interactions are selective. One of the examples of selectivity of such interactions are interactions of the BPs derived from α-helix D of the TIR-domains from TLR with the TIR-domains of the adapter proteins, while these peptides do not interact with the TIR-domains of the receptors.

EFFICIENCY OF BLOCKING PEPTIDES IN *in vivo* APPLICATIONS

The peptide-based blockers of protein–protein interactions identified in the experiments with cell cultures were tested in the *in vivo* experiments. It must be mentioned, however, that at present the reported examples of using BPs *in vivo* are limited to the experiments with small laboratory animals, and in the majority of cases involve testing of TLR inhibitors. In the first attempts to modulate TLR functions *in vivo* with the help of BPs the peptides derived from the viral proteins A46R and A52R were used. Tsung et al. [91] demonstrated that the P13 peptide from the A52R protein decreased the level of circulating TNF induced by administration of LPS to mice almost 2-fold. Similar effect was observed for the VIPER peptide in the case of intravenous administration, which resulted in the decrease of circulating IL-12p40 by ~50% [28]. Couture et al. [80] were the first to test the TLR blocking peptides based on the mammalian proteins in the mouse model. Two peptides identified during the screening of the peptide library of the adapter protein TIRAP were tested; these were peptides from the second and third helical site of the TIRAP sequence, named, respectively, TR5 and TR6. The peptides

were injected intraperitoneally to mice at the dose 10 nmol/g 1 h prior to administration of sublethal dose of LPS. Both peptides, but not the control cell-penetrating peptide of the similar length practically completely blocked influx of TNF into circulation as a response to LPS introduction, and also significantly decreased the level of circulating IL-6 [80].

In the following study, Piao et al. [55] evaluated efficiency of BPs in the animal model in more detail. Inhibitory peptides derived from the adapter protein TRAM belonging to the MyD88-independent signaling pathway were examined [99]. The experiments confirmed systemic inhibitory activity demonstrated by the peptides administered to mice. The peptides TM4, TM6, as well as the truncated peptide TM4-ΔC decreased the levels of circulating TNF and IL-6 by ~90% of their peak levels [55]. The authors compared activity of the peptides administered intraperitoneally and intravenously. The BPs significantly decreased systemic levels of the cytokines in both cases of administration, however, inhibitory activity of the peptides following intraperitoneal administration was higher [55]. Efficiency of BPs was also investigated in the case of so-called "therapeutic administration." Order of administration of BP and LPS was changed in these experiments, BP was administered 30 min after administration of LPS, not one hour prior to that. The experiments demonstrated significant decrease of the levels of circulating cytokines already 1.5 h after "therapeutic administration" of BP [55]. In another series of experiments from the same study the ability of TRAM-peptides to prevent lethality due to administration of LPS to mice was evaluated. Administration of the TM4 and TM4-ΔC peptides at the dose 10 nmol/g one hour prior to administration of LPS at the dose 17.5 μg/g prevented lethal outcome in 100% of cases, while the peptides TM6 and TR6 were effective in ~65-80% of cases [55]. Survival of mice after therapeutic administration of the TM4-ΔC peptide (3 h after administration of the lethal dose of LPS) decreased, as expected, in comparison with the prophylactic administration, and was ~70% versus 100%-survival in the case of the peptide administration 1 h prior to administration of LPS [55].

Use of several variants of the peptides derived from the third helical fragment of the TIRAP sequence has been reported in the literature. In particular, Shah et al. [100] used the peptide MIP2, with eight of 12 amino acid residues in its sequence significantly overlapping with the sequence of TR6, inhibitory peptide identified by Couture et al. [80] in the original screening of the peptide library of the TIR-domain from TIRAP. MIP2 demonstrated multi-specific effect with respect to TLR inhibiting TLR2, TLR3, TLR4, TLR7, and TLR9 [100]. This observation confirmed and expanded the data presented in the Couture et al. study [80], in which effect of TR6 was evaluated only on TLR2 and TLR4. It was shown using the LPS-induced septic shock model that MIP2 increased the 72-h survival of mice from 0 to 20-25% [100]. Shah et al. [100] investigated in detail effects of MIP2 in the models of chronic inflammatory diseases: psoriasis induced by introduction of imiquimod, a TLR7 agonist; lupus (using the MRL/lpr mouse line spontaneously developing this disease); as well as non-alcoholic fatty liver disease (NAFLD) induced by the diet with low content of methionine and choline. The authors reported that the 6-day course of MIP2 had a significant anti-inflammatory effect (similar to the effect of methotrexate) in the psoriasis model, when the peptide was used at a lower dose (1 nmol/g); the effect, however, decreased, when the MIP2 peptide was used at high doses (10 and 20 nmol/g) [100]. In the mouse lupus model, the 20-day course of MIP2 significantly slowed progress of inflammatory symptoms of the disease, while in the model of NAFLD, a prolonged administration of the peptide significantly decreased manifestations of inflammation, although did not decrease the levels of liver markers and histological signs of hepatic steatosis [100]. Another example of the blocking peptide derived from the third helical fragment of the TIRAP sequence is TR667, which represents an evolutionary preserved segment of the surface of TIR-domain differing from MIP2 by the single-amino-acid shift towards the C-end of TIRAP [25]. TR667, similarly to MIP2, exhibited multi-specific inhibitory properties towards TLR and inhibited TLR2, TLR4, TLR5, and TLR9; however, unlike the MIP2 peptide, it did not inhibit TLR7 [25].

Efficiency of the peptide 2R9 with respect to systemic production of cytokines induced by the TLR2 and TLR7 agonists, as well as for suppression of cytokine response to a replication-capable pathogen (PR8 strain of the flu virus H1N1B was used) was evaluated in the study by Piao et al. [56]. The preliminary investigation showed that the peptide 2R9 identified during screening of the peptide library of the TLR2 TIR-domain was multi-specific and blocked TLR2, TLR4, TLR7, and TLR9 due to the binding of TIRAP, adapter protein enhancing signal transduction from these receptors [56]. Administration of 2R9 to mice resulted in significant inhibition of the cytokine response to both Pam3Cys (*S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-Ser-Lys_4-OH), agonist of TLR2, and to R848 (resiquimod), agonist of TLR7, as well as to ODN1668, TLR9 agonist [56, 83]. 2R9 also blocked by 80-90% the MyD88-dependent secretion of TNF, IL-6, and IFN-β by the cultivated peritoneal macrophages infected with the flu virus [56]. Excessive secretion of cytokines could be the cause of lethal outcome in acute viral infections. Based on this consideration the authors tested whether 2R9 could decrease lethality of mice infected with the dose of flu virus (strain PR8)

that causes ~90% lethality. The experiments showed that the 5-day course of daily injections of the peptide starting 48 h after infection with the virus significantly reduced lethality [56]. However, it was shown in another study that the effect of suppression of the TLR-dependent immune response on the outcome of disease significantly depends of the time of the start of the therapy [101], very early administration of the agents suppressing immune response could aggravate the course of infection diseases.

The presented studies demonstrate efficiency of BPs for both suppression of cytokine production induced by introduction of synthetic agonists of specific receptors, as well as in more complex models of chronic inflammation or inflammation induced by infectious agents.

CONCLUSIONS

Studies of recent decades have led to a significant progress in the discovery of new BPs and understanding of mechanisms of their action. At present, techniques for BP identification, evaluation of their binding specificity to targets *in vitro* and in cellular models, as well the methods for evaluation of their efficiency *in vivo* have been established. High efficiency of the methodology of blocking peptides for the development of inhibitors of signaling pathways with mechanisms of action based on blocking transient interaction of signaling proteins realized through the specialized protein domains has been demonstrated. Experiments with small laboratory animals have demonstrated that BPs are capable of suppressing the systemic response to stimulation of certain receptors both in the cases of intraperitoneal and intravenous injections of the peptides. Moreover, there are examples of high efficiency of BPs during prolonged administration for suppression of chronic inflammatory processes. It is recognized that the multi-specificity of binding demonstrated by some BPs is not only important for understanding of their biological effects, but also significantly contributes to their efficacy in complex animal models.

Nevertheless the large number of known BPs, exact molecular determinants of their activity remain to be established. This lack of understanding likely stems from such factors as the diversity of inhibitory sequences, the tolerance of BPs to amino acid substitutions, multiplicity of binding sites of individual BPs, as well as the lack of structural knowledge on the BP-target complexes. Determination of high-resolution 3D-structures of BP complexes with their target proteins should improve our understanding of signaling protein recognition mechanisms, and suggest ways for rational optimization of already known BPs. BPs' efficacy also could be improved via optimization of penetrating sequences for targeted delivery of the inhibitor or via use of peptidomimetics to improve the biological stability of the BPs.

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