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Review

Regulation of protein function by interfering protein species

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Abstract

Most proteins do not function alone but act in protein complexes. For several transcriptional regulators, it is known that they have to homo- or heterodimerize prior to DNA binding. These protein interactions occur through defined protein-protein-interaction (PPI) domains. More than two decades ago, inhibitor of DNA binding (ID), a small protein containing a single helix-loop-helix (HLH) motif was identified. ID is able to interact with the larger DNA-binding basic helix-loop-helix (bHLH) transcription factors, but due to the lack of the basic domain required for DNA binding, ID traps bHLH proteins in non-functional complexes. Work in plants has, in the recent years, identified more small proteins acting in analogy to ID. A hallmark of these small negative acting proteins is the presence of a protein-interaction domain and the absence of other functional domains required for transcriptional activation or DNA binding. Because these proteins are often very small and function in analogy to microRNAs (meaning in a dominant-negative manner), we propose to refer to these protein species as 'microProteins' (miPs). miPs can be encoded in the genome as individual transcription units but can also be produced by alternative splicing. Other negatively acting proteins, consisting of more than one domain, have also been identified, and we propose to call these proteins 'interfering proteins' (iPs). The aim of this review is to state more precisely how to discriminate miPs from iPs. Therefore, we will highlight recent findings on both protein species and describe their mode of action. Furthermore, miPs have the ability to regulate proteins of diverse functions, emphasizing their value as biotechnological tools.

Keywords: homotypic interaction; interfering protein; microProtein; protein-protein interaction; transcription factor inactivation.

Introduction

The function or activity of proteins is often regulated at the posttranslational level. Regulation can occur *via* modification of the protein sequence (e.g., phosphorylation or ubiquitination) or *via* interaction with other proteins to form either a functional or a non-functional protein complex. A prerequisite for proteins to form functional complexes is the presence of protein-protein-interaction (PPI) domains. Transcriptional regulators are often active as dimers, and the interaction occurs through protein-dimerization domains such as leucine-zipper or zinc finger motifs. Owing to the requirement of some transcription factors to be active as homodimers, protein species were identified that specifically interfere with dimer formation by sequestering these transcription factors into non-functional protein complexes (1–3). A recent review on these interfering protein (iP) species proposes to refer to these as small interfering peptides (4). Because peptides are commonly known to be rather short (in the order of as few as 2–50 amino acids) and often derived from cleavage of precursor proteins, we feel that the name is somewhat misleading. Therefore, we propose to refer to these protein species as iPs and, in the case of small, single-domain interfering species, as microProteins (miPs).

miPs are small iP species, which perturb the formation of protein complexes of the targets they regulate (5). Both miPs and their targets harbor a highly similar PPI domain, and the negative effect is due to the formation of heterodimeric miP/target protein dimers. MiPs consist of only a single PPI domain and are either encoded as single entities in the genome or can be produced by means of alternative splicing.

MiPs that are encoded in the genome, evolved most likely during genome-duplication events followed by domain loss. However, this process, as well as alternative splicing, can also yield negatively acting protein species that harbor, besides the PPI domain, other functional domains. Here, we would like to further concretize the miP concept and discriminate between genuine miPs and other iP species (Figure 1).

So far, all miPs described to date target transcriptional regulators. However, it is conceivable that also other proteins, which are functional as multimers, can be targeted by miPs.

Plant miPs involved in the regulation of stature

The first miP, the helix-loop-helix (HLH) protein inhibitor of DNA binding (ID), was identified more than two decades ago in mice (6). By interacting with basic helix-loop-helix (bHLH) transcriptional regulators, *via* the HLH domain, ID can sequester these into non-functional heterodimeric complexes (6). In mice, ID is involved in the regulation of muscle differentiation. By interacting with two ubiquitously expressed bHLH transcription factors,

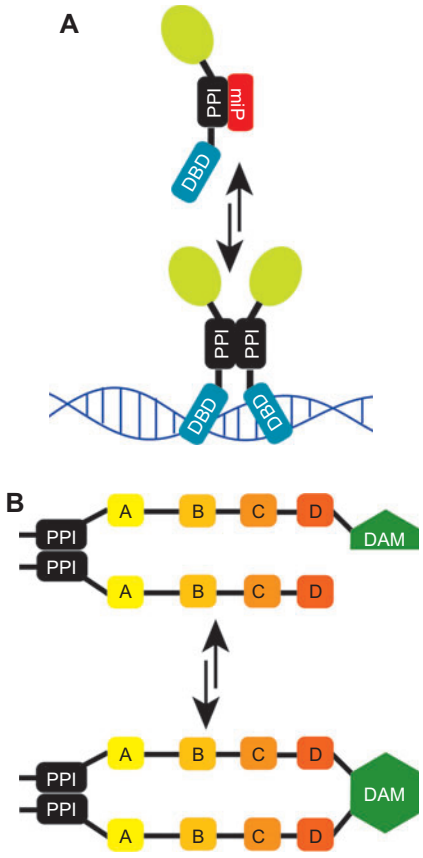

Figure 1 Discrimination between miPs and iPs. (A) miPs consist of a single PPI domain, allowing them to heterodimerize with their targets. It can be assumed that equilibrium between miP-inhibited protein complexes and homodimeric active complexes exists. (B) iP species can be encoded in the genome or produced by alternative splicing. They consist of multiple domains, including a PPI domain allowing them to heterodimerize with their targets. In case the target protein contains a domain, which requires to homodimerize in order to be active [dimeric active motif (DAM)], short iP species can negatively interfere with the formation of active protein complexes.

E47 and E12, ID allows MyoD, a bHLH protein as well and master regulator of muscle differentiation, to dimerize and exert its function (7, 8). Since the identification of ID in mice, several other ID-like proteins have been identified in animals being involved in the regulation of cell fate in different tissues (9–12) and the regulation of the circadian clock (13). ID-like proteins also exist in plants, but phylogenetic analysis indicates that plant-like ID proteins evolved independently (5). Like their counterparts in the animal kingdom, plant ID proteins also act by sequestering bHLH transcription factors into non-functional complexes. In plants, ID-like proteins have been shown to be involved in brassinosteroid hormone signaling (14, 15) and the regulation of shade growth.

The first identified plant-specific miPs are the LITTLE ZIPPER (ZPRs, small leucine zipper proteins) proteins, which are involved in the maintenance of the shoot stem cells and polarity establishment in developing leaves (1, 2). ZPRs are small leucine-zipper proteins and act by forming non-functional heterodimeric complexes with class III homeodomain leucine zipper transcription factor family (III HD-ZIP). Class III HD-ZIPs are evolutionary highly conserved (16) and are key regulators of polarity processes in plants. A concept review on plant and animal miPs was published recently (5). In the following, we will provide further insight on recently identified miPs and their roles in the regulation of developmental processes.

All plant miPs found to date act on transcriptional regulators and perturb DNA binding by forming non-functional heterodimeric complexes (Figure 1A) (5). However, it is imaginable that miPs can also act by retaining transcriptional regulators in the cytoplasm. This has recently been shown for the MINI FINGER1 (MIF1), a miP containing a zinc-finger domain, required to heterodimerize with zinc finger homeodomain protein 5 (ZHD5). The *MINI FINGER* (*MIF*) gene family comprises three genes in *Arabidopsis*-encoding MIF miPs. Initially, they were identified in an activation-tagging approach, and dominant overexpression plants exhibited largely pleiotropic phenotypes, such as dwarfism, lost apical dominance, altered leaf and flower development, and constitutive morphogenic responses (17). Phylogenetic analysis of *ZHD* and *MIF* genes revealed that *ZHD* genes are plant-specific transcription factors, which can also be identified in lower plants such as mosses (18). During genome duplication events, *MIF* genes evolved, encoding proteins lacking the homeodomain. *MIF* genes are only found in higher plants such as Gymnosperms and Angiosperms (18). Even though the phylogenetic relationship between ZHD and MIF protein was recognized, the mode of action of MIF proteins was just recently discovered (3). In contrast to overexpression of MIF1 protein that causes dwarfism, overexpression of ZHD5 results in plants with increased stature, when compared to wild-type non-transgenic plants (3). Interestingly, besides acting like the other plant miPs and preventing ZHD5 from DNA binding, MIF1 most likely enhances its dominant-negative potential by additionally sequestering ZHD5 in the cytoplasm (3). Thus, MIF-type miPs act at two levels to keep ZHD target proteins in check. How expression of the MIF/ZHD modules is regulated and under which conditions MIF-type miPs are most active still remains elusive.

miPs can act non-cell autonomously

A common theme in both plant and animal development is the regulation of developmental processes by non-cell-autonomous factors. These factors are produced in one cell and then transported to neighboring cells, where they exert their function. In the case of small RNAs, it was shown that they often act in a non-cell-autonomous systemic manner (19). In animals, proteinaceous systemic signals are well-known, since many hormones are transported *via* the blood stream and act at places that are distant from the place of production. Plants also produce proteins that are transported *via* the vasculature over long distances. Important to note

here are systemin, a short peptide produced in response to pathogen attack (20), or the recently identified flowering trigger florigen, which comprises the small FLOWERING LOCUS T protein (21). Besides acting in a systemic manner, some proteins act locally but non-cell autonomously. Lateral inhibition is a process in which one cell produces a signal that moves to the neighboring cells to suppress certain cell fates. This results in the manifestation of biological patterns, as seen for sensory bristle spacing in *Drosophila* (22), or the correct organization of the neural chord in higher animals (23).

In order to be able to act in a non-cell-autonomous manner, a protein needs to fulfill certain criteria, the most important being size. It is assumed that proteins smaller than 40 kDa are able to move freely between cells by diffusion, but for increased transport rate or directional movement, cofactors are required (24). As a rule of thumb, one could say that the smaller the size of a protein, the more likely this protein can move between cells. Since miPs are very small (in the case of the smallest functional ZPR protein around 70 amino acids, corresponding to around 8 kDa), it seems possible that most of them are able to act outside the cells in which they are produced. In the following, we will highlight two examples of miPs acting non-cell autonomously.

Trichomes, hairs growing on the leaf blade, as well as root hairs, are derived from epidermal precursor cells, the so-called trichoblasts. An intricate patterning network, involving transcription factor movement between cells, controls the development of trichoblasts and thus the positioning and spacing of hair cells on the roots and leaves (25, 26). Trichome patterning involves three evolutionary highly conserved positive regulators comprising a WD40 protein, several bHLH transcription factors, and R2R3-MYB transcription factors. The latter have been shown to be competitively inhibited by R3-type MYB miPs. The *Arabidopsis* genome encodes seven R3MYB-type miPs, and all of them have been shown, when overexpressed, to act in a dominant-negative manner on their targets, the R2R3-type MYB proteins. Furthermore, molecular and theoretical modeling approaches have revealed that the inhibitory role of the individual R3MYB miPs differs among the seven members and that this divergence is due to an unequal cell-to-cell movement ability (27). Furthermore, the efficiency with which R3MYB-type miPs move between cells also depends on the levels of miP target proteins (27). Thus, multiple parameters influence the lateral inhibitory effect of R3MYB-type miPs.

Plant ID-like HLH-type miPs have recently been shown to interfere with the brassinosteroid signaling pathway and control growth responses both in *Arabidopsis* and rice (14, 15). These miPs, named PRE, interact, like the animal ID counterparts, with bHLH proteins and thereby influencing the ability of the protein complex to bind DNA. The analysis of targets of the MONOPTEROS protein, an auxin response factor-like transcription factor involved in the regulation of early embryo patterning of *Arabidopsis*, yielded the identification of PRE3, an HLH miP, now named TARGET OF MONOPTEROS7 (TMO7) (28). A difference between the mRNA expression domain (based on a TMO7 promoter fragment driving the expression of a nuclear localized GFP) and the protein expression domain (based on a TMO7 promoter fragment driving the expression of a TMO7-GFP fusion protein) was observed. Interestingly, TMO7-GFP was able to only move from its place of expression (lower half of the developing embryo) into the hypophysis but not to the apical half of the embryo, suggesting a directional process (28). In addition, TMO7 localizes both to the nucleus and the cytoplasm suggesting a two-layered regulation of potential target proteins: First, target bHLH proteins are sequestered into non-functional complexes, and second, inactive complexes are retained in the cytoplasm.

Alternative splicing as a source for miP and iP generation

In the previous section, we have described examples of miPs and iPs encoded in the genome as independent transcription units. Another potential source for generating miPs is alternative splicing. Alternative splicing is the process of generating several mRNA isoforms from one premature mRNA by using different combinations of available exons. This process involves exon skipping, intron retention, or the use of alternative 5′- and 3′-splice sites. In the last years, it has been shown that the majority of the genes in higher organisms containing more than one intron undergo alternative splicing (29), and it is suggested that alternative splicing makes a significant contribution to proteome diversity in higher eukaryotes (29). Alternative splicing has the potential to give rise to miPs and iPs by skipping or splicing of exons coding for protein domains necessary for correct function. For example, alternative splicing of a transcription factor, which requires homodimerization to be active, can be regulated by a splice variant lacking the DNA-binding domain but containing the domain required for mediating protein-protein interactions. The truncated transcription factor will dimerize with the full-length transcription factor, but the dimer will not be able to activate transcription. Several examples of such miPs and iPs derived from alternative splicing have been identified, most of them in animals (30–36). One of the first described examples of alternative splicing-generated miPs is the murine TFE3 bHLH leucine zipper transcription factor. Roman et al. identified a splice variant (TFE3-S) lacking 35 amino acids comprising the transcriptional activation domain (31). The authors were able to show that the short splice variant is able to interact with DNA, to form homodimers with itself and to heterodimerize with the full-length TFE3 protein (TFE3-L). However, the ability of transcriptional activation of the TFE3-S homodimers and the TFE3-S-TFE3-L heterodimers is significantly reduced (31). It was further shown that both splice variants occur in different tissues in dissimilar ratios and that low amounts of TFE3-S are sufficient for a significant reduction of the TFE3-mediated transcriptional activation (31). The short splice variant of TFE3 is, with a size of 537 amino acids,

only 35 amino acids shorter than the functional full-length TFE3-L, and it contains several protein domains. According to our previous definition, TFE3-S resembles an iP, generated by alternative splicing.

An interesting example for a genuine miP derived from alternative splicing is a recently described splice variant of the plant bHLH transcription factor phytochrome interacting factor 6 (PIF6), belonging to the PIF protein family. *PIF*s comprise a group of seven genes in *Arabidopsis*, and PIF proteins directly interact with the phytochrome-type red-light sensing photoreceptors. In higher plants, phytochromes are involved in the regulation of developmental processes like seed germination, phototropism, and shade avoidance (37). PIFs play a central role in these light-dependent developmental processes. Previously, proteins have been identified regulating the function of PIFs. For example, HFR1, an atypical bHLH transcription factor, acts by binding PIF4 and PIF5, trapping them in non-functional complexes (38). In addition, HFR1 is itself regulated by the miP KDR, which binds HFR1 and prevents its binding to PIF4 or PIF5, increasing the ability of PIF4 and PIF5 to homodimerize (39).

PIF6 was identified in a screen for mutant plants with germination defects (35). It is expressed during seed development and shows highest expression levels in dried seeds. Expression of PIF6 is rapidly reduced after exposure of dry seeds to water. In *Arabidopsis*, two splice variants of PIF6 exist, one full-length variant (named PIF6-á) containing the bHLH domain necessary for the formation of protein dimers, DNA binding, and transcriptional activation, and an APB domain required for phytochrome interaction. The short splice variant PIF6-â lacks the HLH domain and contains only the motifs necessary for the interaction with phytochromes (35). It was shown that the *pif-6* knock-out mutants show a reduced germination rate under high temperatures (35). Overexpression of PIF6-â, however, increases germination at higher temperatures. Overexpression of both splice variants has a slight effect on hypocotyl elongation under red light. Penfield et al. suggest that only the PIF6-â splice variant affects primary dormancy in seeds, whereas the full length variant PIF6-á does not seem to have a significant function, and they assumed that the effects of PIF6-â are due to its interaction with phytochromes (35). This seems plausible, but further research is required to fully understand the role of both PIF6 splice variants in the phytochrome signaling network.

Another recently described example for an iP in plants derived by alternative splicing is the short â variant of indeterminate domain 14 (IDD14), a C2H2 zinc-finger-type transcription factor, which requires dimerization for DNA binding and transcriptional activation (36). The full-length IDD14á is a protein of 419 amino acids containing three zinc-finger motifs. It activates expression of QQS, a protein that regulates starch accumulation in the leaves of *Arabidopsis thaliana* (40). The IDD14â variant, however, contains only two zinc-finger domains and is 86 amino acids shorter than IDD14á. Overexpression of IDD14â in transgenic plants causes a phenotype similar to *idd14-1* mutant plants, viz. accelerated growth and slightly earlier flowering. Conversely, plants overexpressing IDD14á grew slower and flowered later than wild-type plants. IDD14á forms homodimers, which bind to the promoter of QQS, while IDD14â homodimers do not. Several experiments revealed the formation of homodimers by IDD14á and IDD14â and also heterodimerization between both splice variants. Double mutant plants, overexpressing both IDD14 variants, displayed no obvious mutant phenotypes leading Seo et al. to assume that IDD14â forms non-functional heterodimers with IDD14á. In wild-type plants, the short splice variant IDD14â is preferentially generated under cold temperatures, which correlates with decreased expression of *QQS* under those conditions (4, 36).

Potential miPs regulating non-transcription factor proteins

Sequencing genomes has yielded the identification of protein-coding sequences. These proteins (many of them still of unknown function) are involved in a plethora of biological processes, and through means of alternative splicing, proteome diversity can be further increased. Another finding is that proteins seldom function alone. By interacting with other proteins, often forming larger protein complexes, the ability of proteins to execute biological functions is further increased. The capability of proteins to form complexes requires protein interaction domains. Different interaction domains can mediate a variety of interactions such as protein-protein interaction, protein-hormone interaction, or protein-RNA interaction. In the following, we will describe and highlight potential miPs targeting other proteins then transcription factors.

RNA-binding proteins as potential miP targets

Several proteins from distinct families have the ability to interact with RNA. RNA-binding proteins are involved in processes such as the regulation of RNA localization, RNA transport, RNA stability, splicing, and translation. The Pumilio family of proteins contains multiple Puf repeats that are able to bind RNA (41). The founding member, the *Drosophila* Pumilio protein is involved in the establishment of anterior-posterior polarity in the *Drosophila* embryo (42) and functions by binding to *hunchback* mRNA, thereby repressing its translation (43). A recent survey of *Arabidopsis* Pumilio-like PUF proteins revealed the existence of two family members, AtPUM25 and AtPUM26, which harbor only three to four Puf repeats and no other domains (41). AtPUM25 and AtPUM26 are also very small in size (137 and 187 amino acids) and thus could function as potential miPs. If the interaction between Puf proteins and their target mRNAs is very specific, they could function by sequestering, and thereby stabilizing, mRNA targets. This, however, needs to be experimentally verified.

Ion channels as potential miP targets

An example for a miP inhibiting the function of an ion channel is the Vpu protein, an accessory protein of human immunodeficiency virus (HIV). Vpu is a small transmembrane protein that consists of 82 amino acids. It promotes the release of virus particles from infected cells and the degradation of CD4, a coreceptor of the MHCII system on the surface of antigen-presenting cells. The function of Vpu is based on its ability to interact with host molecules. In infected cells, it leads to the rapid degradation of CD4 by forming a complex with CD4 and TrCP, a component of SCF^{TrCP} E3 ubiquitin ligase complex. In addition, Vpu can also function as a miP because it shows a high degree sequence conservation with the N-terminal region of the human K^+-ion channel TASK-1 (44). This homologous region has been shown to be required for channel multimerization (45). When expressed as a single domain, it is capable of oligomerization with the full-length voltage-dependent K^+ channel, resulting in the formation of non-functional ion-channel complexes (46). Hsu et al. compared Vpu and the single domain of TASK-1 (Ttm1) in their ability of regulating TASK-1 function (44). The authors could show that both Vpu and Ttm1 interact with TASK-1 and inhibit its K^+ conductivity. Interestingly, both proteins also promote the release of HI-Viruses from the host cell, so it seems that TASK-1 is detrimental for this process. Vpu also reduces the amount of TASK-1 in the cell in a similar way as it reduces the amount of CD4, by recruiting TrCP resulting in ubiquitination and subsequent proteasomal degradation (44). The exact role of TASK-1 in virus reproduction and detachment is not yet defined, but the inhibition of TASK-1 through Vpu *via* two different ways, the formation of non-functional multimers and the induced degradation, underlines the necessity of this process.

Vpu is not the only example for a small protein, only consisting of a single transmembrane domain, regulating a K^+ channel. A similar regulatory mechanism has been described for fast activating voltage-gated potassium channels (Kv). There is a broad variability of these channels expressed in various tissues, especially in fast spiking neurons, where these channels allow a fast recovery after a depolarization (47). The á subunits Kv3.1 and Kv3.2 show a very low deactivation rate, which allows a rapid regeneration of the membrane potential in this fast spiking neurons. The Kv3.1 and Kv3.2 á subunits can also be found in non-fast spiking neurons and other tissues. In these cell types, they are often associated with MinK, MiRP1, or MiRP2, small proteins of 129, 123, and 103 amino acids, containing only one transmembrane domain, homologous to the first transmembrane domain of Kv á subunits, which is necessary for multimerization. MinK, MiRP1, and MiRP2 can interact with other Kv subunits to form heteromers with K^+ channels, but these channels respond and recover slower than channels not containing these miPs (47, 48). This example highlights that miP interference does not always result in a complete suppression of target protein function but enables a fine tuning of protein complexes they are interacting with. These naturally occurring miPs illustrate the application of artificial miPs as tunable regulators of protein complex function.

Biotechnological aspects

Cellular signaling processes largely rely on the formation of active complexes through protein-protein interactions. miPs can interfere with the formation of functional dimers by forming non-functional protein complexes. The ability of a miP to interfere with potential targets is determined by different parameters: the dissociation constants of both the active homodimer as well as the inactive heterodimer and the concentrations of both target protein and miP. By binding to target proteins, miPs have the ability to evoke ultrasensitive responses. Ultrasensitivity is the process of excessive inhibition by only moderately altering the concentration of inhibitor (here: miP) (49, 50). By using different promoters (e.g., cell type specific with high expression, cell type specific with low expression, or inducible systems) to drive miP expression, ultrasensitive responses can be elicited (49, 50). Furthermore, by experimentally designing artificial miPs, it might be possible to create protein variants that interact strongly with their targets in addition to variants that bind only weakly or even transiently. Small protein variants, which selectively inactivate target proteins and whose action can be tuned by modifying expression level, will certainly be valuable tools in synthetic biological applications as they allow establishing novel regulatory circuits. The use of directed evolution approaches, as shown for the identification of potential ligands for yeast Src homology 3-domain proteins (51) will allow the identification of synthetic miPs with either high or low specificity for their targets.

Outlook

miPs are potent regulators of protein activity in both plants and animals. They act by sequestering target proteins into non-functional complexes, and some can function in a non-cell-autonomous manner. miPs can exist as individual genes but can also be produced by alternative splicing. It might be possible that miPs can also be generated through proteolytic cleavage of proteins, thereby losing domains required for activity but retaining domains required for dimerization. Examples for miPs/iPs generated by proteolytic cleavage are the CCAAT displacement protein and the serum response factor in animals (more details can be found in the recent review by Seo and colleagues (4).

Most published examples of miPs target transcription factors, but here, we have shown examples of potential miPs targeting non-transcription factor proteins. It is conceivable that it is possible to generate artificial miPs targeting proteins of various function. We have mentioned examples of ion channel regulation (Figure 2A), and it seems possible to create miPs harboring other functional domains such as hormone-binding sites (Figure 2B). By overexpressing artificial miPs encoding hormone receptor domains, it might be possible to sequester the hormone, thereby depleting its effect. All miPs targeting transcription factors act by trapping the active protein into non-active heterodimeric complexes. However, not all transcription factors have

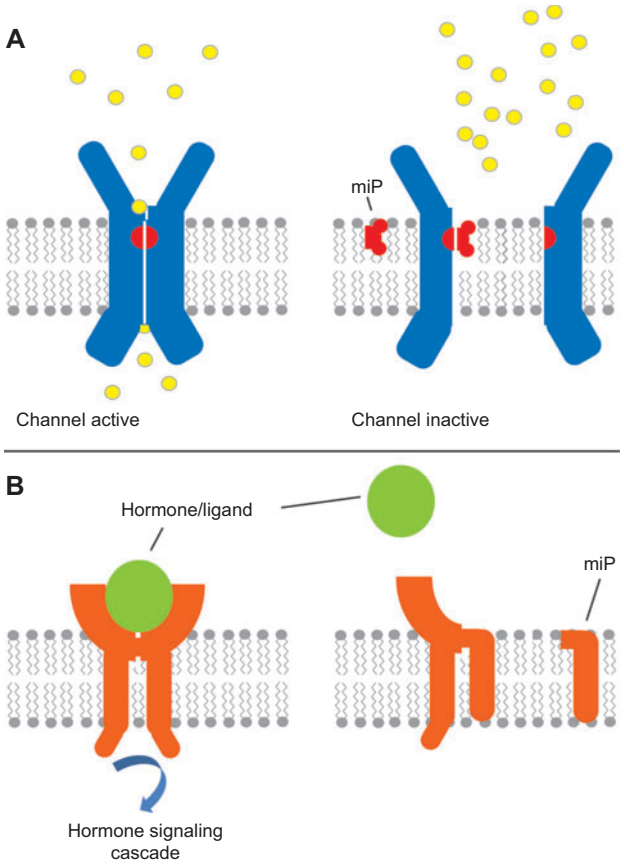

Figure 2 miPs interfering with non-transcription factor proteins. (A) Regulation of ion channel activity. This example shows a protein dimer forming a functional pore. Dimerization is mediated through a PPI domain (red). miPs containing the PPI domain can sequester the proteins into non-functional heterodimeric complexes, perturbing pore formation and channel function. (B) Potential regulation of membrane-bound hormone receptors. MiP proteins form non-functional heteromers thereby blocking hormone signaling.

protein-interaction domains and might thus regulate transcription as monomers. Knowing the *cis*-elements these monomeric regulators bind to, it might also be possible to generate artificial miPs comprising only the DNA-binding domain. These artificial miPs might be able to bind DNA and block the sites of their 'targets'.

All true plant miPs that have been identified in the past 5 years (4, 5) have appeared relative recent and are mostly found in angiosperm species. This finding suggests that they are most likely a by-product of genome duplication events. Functional domains were lost, enabling these protein species to acquire a new, negative role as protein sequestration factors. Pathogenic viruses often integrate their genome into the host genome, which can cause a transfer of host DNA into the virus particle. This process is prone to create new miPs, as the amount of genetic material transferred to the virus is limited. An indication that this process has already occurred in nature is the small protein channel regulator Vpu1 of HIV. This finding illustrates that by mining proteomes of pathogenic viruses, bacteria, or yeast, novel miPs can be identified that strongly interact with host proteins because they originated from the host genome.

Highlights

- miPs are small, single-domain proteins able to interfere with larger proteins harboring a similar domain required for the formation of protein dimers.
- Negative interference is often the result of the formation of non-functional protein dimers.
- miPs can exist as individual transcription units in the genome but can also be produced by alternative splicing.
- Another class of negative regulators, we refer to as 'iPs' exist. A hallmark of these proteins is that they contain more than one functional domain and thus could also control more than one biological process.

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