New ribosome-inactivating proteins and other proteins with protein synthesis-inhibiting activities

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Abstract

Ribosome-inactivating proteins (RIPs) consist of three varieties. Type 1 RIPs are single-chained and approximately 30-kDa in molecular weight. Type 2 RIPs are double-chained and composed of a type 1 RIP chain and a lectin chain. Type III RIPs, such as maize b-32 barley and JIP60 which are produced as single-domain proenzymes, possess an N-terminal domain corresponding to the A domain of RIPs and fused to a C-terminal domain. In addition to the aforementioned three types of RIPs originating from flowering plants, there are recently discovered proteins and peptides with ribosome-inactivating and protein synthesis inhibitory activities but which are endowed with characteristics such as molecular weights distinctive from those of the regular RIPs. These new/unusual RIPs discussed in the present review encompass metazoan RIPs from Anopheles and Culex mosquitos, antimicrobial peptides derived from RIP of the pokeweed Phytolacca dioica, maize RIP (a type III RIP derived from a precursor form), RIPs from the garden pea and the kelp. In addition, RIPs with a molecular weight smaller than those of regular type 1 RIPs are produced by plants in the Cucurbitaceae family including the bitter gourd, bottle gourd, sponge gourd, ridge gourd, wax gourd, hairy gourd, pumpkin, and Chinese cucumber. A small type II RIP from camphor tree (Cinnamomum camphora) seeds and a snake gourd type II RIP with its catalytic chain cleaved into two have been reported. RIPs produced from mushrooms including the golden needle mushroom, king tuber mushroom, straw mushroom, and puffball mushroom are also discussed in addition to a type II RIP from the mushroom Polyporus umbellatus. Bacterial (Spiroplasma) RIPs associated with the fruitfly, Shiga toxin, and Streptomyces coelicolor RIP are also dealt with. The aforementioned proteins display a diversity of molecular weights, amino acid sequences, and mechanisms of action. Some of them are endowed with exploitable antipathogenic activities.

Introduction

Ribosome-inactivating proteins are characterized by the ability to inhibit protein synthesis. They have drawn a great deal of attention because of the plethora of exploitable activities including anticancer, antifungal, antibacterial, antiviral, and insecticidal activities (Stirpe and Lappi 2014; Akkouh et al. 2015; Zhu et al. 2018). Ribosome-inactivating proteins (RIPs) comprise type I RIPs which are single-chained proteins with a molecular weight in the vicinity of 30 kDa and type II RIPs composed of a type 1 RIP chain and a lectin chain (Fabbrini et al. 2017; Zhu et al. 2018).

Type I and type II RIPs may co-exist in the same plant tissue (de Benito et al. 1995). Type II RIPs consist of both toxic and nontoxic proteins. Though nontoxic RIPs have lower anticancer activity, their conjugates with other molecules can be used to target against cancer (Girbés et al. 1993; Girbes et al. 2003; De Benito et al. 1995; Muñoz et al. 2001; Citores et al. 2002). The list of unique RIPs covered in this review include those from mosquitos and bacteria, the kelp Laminaria japonica, the gymnosperm cypress tree, maize (a type 3 RIP derived from a precursor), peptides prepared from Phytolacca RIP, garden pea RIP, small RIPs from flowering plants in the Cucurbitaceae family, a small type II RIP from camphor tree (Cinnamomum camphora) seeds, a type II RIP from the mushroom Polyporus umbellatus, mushroom RIPs, a type 2 RIP from snake gourd seeds with a cleaved catalytic chain, and bacterial RIPs including Spiroplasma RIP, Shiga toxin and Streptomyces coelicolor RIP. The aim of this article is to review new types of proteins with ribosome-inactivating and protein synthesis inhibitory activities.

Mosquito RIPs

Culex quinquefasciatus RIP and Aedes aegypti RIP

Genes coding for RIPs in the mosquito species C. quinquefasciatus and A. aegypti have been described. Results of assessment of taxonomic distribution, phylogenetic inferences, and microsynteny analyses suggest the origin of mosquito RIP genes from a single horizontal gene transfer (HGT) event, presumably from a cyanobacterial donor. Evolutionary analyses disclose that, subsequent to the HGT event, the mosquito RIP genes evolved under purifying selection, implying functional roles in mosquitos (Lapadula et al. 2017).

Algal RIP

Lamjapin

Lamjapin, a 36-kDa type I RIP from kelp (L. japonica A), is the first RIP reported from a marine alga. It exhibited cell-free protein synthesis in the inhibitory activity in rabbit reticulocyte lysate with an IC50 of 0.69 nM. It was capable of cleaving purine at multiple sites of RNA in the rat ribosome to yield the diagnostic R-fragment and three bigger fragments following addition of aniline. Lamjapin split the adenyl residue at the site A20 of the 35-mer synthetic oligoribonucleotide substrate used to simulate the sarcin/ricin domain in 28S RNA from rat ribosomes. It was incapable of catalyzing the hydrolysis of the N-C glycosidic bond in cytidine, guanosine, or uridine at the corresponding site of the A20 of three mutant sarcin/ricin domain RNAs (Liu et al. 2002, 2017).

Gymnosperm RIP

Biota orientalis RNase

The 13-kDa B. orientalis RNase (RNase Bo) from the mature cypress tree (Oriental arborvita) seeds produced cleavage of a phosphodiester linkage between C4453 and A4454 referred to as B. orientalis RNase region of 28S RNA in rat ribosomes in the presence of magnesium ions at a concentration of 25 mM. A small RNA-fragment called the S-fragment was generated and this action resembled that of α-sarcin. The distance between the cleavage-site of α-sarcin (G4325) was at a distance of 128 nucleotides away from that of B. orientalis RNase (C4453). The damaged ribosome failed to bind aminoacyl-transfer RNA (Xu et al. 2004; Liu et al. 2017).

Flowering plant type III RIP

Maize RIP

The active heterodimeric form of maize RIP was composed of a16.5-kDa chain and an 8.5-kDa chain. It was derived from an inactive 34-kDa precursor exhibiting an isoelectric point of 6.5, by removing from the center of the precursor, a 25-residue internal inactivation domain carrying a net charge of − 6, to yield a form exhibiting an isoelectric point above 9 and subsequent processing at the C and N terminals. This is a unique way of precursor processing since it usually involves cleavage at the N terminal (Walsh et al. 1991).

The susceptibility of maize ribosomes to pokeweed RIP was considerably higher than that to the precursor and active forms of maize RIP, indicating the precursor form of maize RIP does not serve as a protective device for shielding homologous ribosomes from attack by the active forms of maize RIP. The 25-amino acid inactivation region, and not the extensions at the N- and C-terminals, mainly accounts for the inhibition of enzymatic activity in the precursor form of maize RIP. Elimination of all three aforementioned regions resulted in activity similar to the active form of maize RIP (Hey et al. 1995).

Recombinant maize proRIP1 (rproRIP1) was devoid of enzymatic activity against ribosomes of various species. Compared with an active deletion mutant of rproRIP1, rproRIP1 activated by papain expressed higher activity toward rabbit and Aspergillus flavus ribosomes, especially in the case of rabbit ribosomes. rproRIP1 activated by papain exhibited substantially higher activity toward rabbit ribosomes than maize ribosomes and was devoid of enzymatic activity toward E. coli ribosomes (Krawetz and Boston 2000).

Maize kernel ribosome-inactivating protein 1 (RIP1) but not RIP1 mutant protein devoid of ribosome-inactivating activity exerted a potent antiproliferative action on nonpathogenic Aspergillus nidulans hyphae. RIP1 induced hyphal branching in pathogenic A. flavus with only a single growing hyphal tip in one conidium. RIP1 inhibited the postdivisional growth stage in both A. nidulans and A. flavus (Nielsen et al. 2001).

Transgenic rice plants expressing both a modified maize RIP gene and a rice basic chitinase gene were more resistant to sheath blight fungus Rhizoctonia solani, but were protected from the fungi Bipolaris oryzae and Magnaporthe grisea (Kim et al. 2003).

Transgenic tobacco (Nicotiana tabacum) plants overexpressing both activated maize RIP and tobacco anionic peroxidase which are resistance proteins were more resistant to feeding by larvae of the cigarette beetle Lasioderma serricorne and the corn earworm Helicoverpa zea and caused a higher larval death rate than wild-type tobacco plants (Dowd et al. 2006).

In addition to maize RIP1, there is a second maize RIP (RIP2), coded for by the gene Rip3:2. Rip3:2 RNA was found in low levels in leaves, shoots, roots, silks, and tassels. Distinct from the Rip3:1 gene, the Rip3:2 gene is not regulated by the transcriptional activator Opaque-2 but upregulated when there is drought. RIP2 resembles proRIP1 in general except for a 19-amino-acid internal portion of proRIP2 which bears little resemblance to proRIP1 other than abundance in acidic amino acids. Distinct regulation of Rip3:1 and Rip3:2 genes indicates that both RIP1 and RIP2 are defense proteins but RIP1 is regulated developmentally whereas RIP2 is influenced by the environment (Bass et al. 2004).

Crystals of the active form and the proenzyme form of maize RIP have been diffracted to 2.5 and 2.4 A, respectively, with main chain root mean square deviation (RMSD) of 0.519. In the proenzyme form, the 25-residue internal inactivation domain is located superficially and comprises a flexible loop succeeded by a long alpha-helix. Interaction of maize RIP with ribosome and its cytotoxicity, but not cellular uptake, was reduced in the presence of the internal inactivation domain. Maize ribosome-inactivating protein resembles its bacterial counterparts in lacking a back-up glutamate at the active site, which assists the protein to keep some residual activity in the event of a mutation of the catalytic glutamate. The structure disclosed that the active site is not large enough to house a pair of glutamate residues. The maize ribosome-inactivating protein may be an intermediate during the course of evolution RIPs (Mak et al. 2007).

During germination, proteolytic cleavage of the 25-amino acid internal inactivation region leads to the formation of the active heterodimeric maize RIP for protection against invading organisms. HIV-1 protease–activated maize RIP variants produced in vitro manifested augmented N-glycosidase activity and suppressive activity on p24 antigen production in HIV-1-infected human T cells in comparison with maize RIP which had not been activated (Law et al. 2010).

Maize RIP is similar to type I RIPs and the A-chain of type II RIPs in protein structure in general. However, it differs from them in some areas such as shorter alphaB and beta 6 segments for facilitating substrate binding, and an alpha-helix in lieu of an antiparallel beta-sheet in the C-terminal region implicated in binding ribosomal protein P2 in certain RIPs. In addition there is relocation of P2 binding site to the N-terminal region close to the internal inactivation domain due to changes in the electrostatic surface potential and three-dimensional structures on the protein (Yang et al. 2010).

Mature maize leaves expressing maize ribosome-inactivating protein and wheat germ agglutinin were more resistant to feeding by first-instar larvae of fall armyworms (Spodoptera

frugiperda) and corn earworms (H. zea) and to Fusarium verticillioides. Maize ribosome-inactivating protein and wheat germ agglutinin neither synergized with nor antagonized one another in their anti-insect activity (Dowd et al. 2012).

Hydrophobic interaction of maize RIP with the 'FGLFD' motif of the ribosomal stalk protein P2 through Lys158-Lys161 found in the N-terminal region and at the base of its internal loop was attenuated compared with Shiga toxin A and trichosanthin. The dissociation constants of the interaction were 195 µM with Shiga toxin A, 612 µM with trichosanthin and 1038 µM with maize RIP, respectively. The cytotoxicity of maize RIP toward 293T cells and its ribosome depurinating activity were 10 times and 48 times lower than those of trichosanthin, respectively (Wong et al. 2012).

Maize ribosome-inactivating protein 2 (RIP2) is produced as an inactive precursor form which is proteolytically cleaved in the gut of fall armyworm (S. frugiperda) larvae to yield the active form. RIP2 is a defense protein which protects the maize plant from insects. Larval feeding, rather than mechanical wounding, brought about an excessive level of RIP2 in the leaves. Plant hormones such as abscisic acid, ethylene, and methyl jasmonate regulated the expression of RIP2 (Chuang et al. 2014).

Recombinant active maize RIP inhibited ex vivo lysis of macaque peripheral blood mononuclear cells which had been infected with chimeric simian-human immunodeficiency virus (SHIV) 89.6. The viral load in plasma was lowered transiently. Adverse changes in immune function and untoward side effects were indiscernible, rendering maize RIP a promising candidate for development into an anti-HIV therapeutic agent (Wang et al. 2015).

The two cereal RIPs, maize RIP1 and barley JIP60, and probably also sorghum RIP, differ from other plant RIPs because of their proteolytic activation mechanisms and absence of signal peptides. Cereal RIPs likely shield the plants from attack by insects and fungal pathogens and may play a role in abiotic stress response or senescence (De Zaeytijd and Van Damme 2017).

Peptides derived from flowering plant type I RIP

Peptides from leaves of type 1 RIP Phytolacca dioica

Peptide PDL440-65, derived by chemical fragmentation of P. dioica type 1 RIP PD-L4, was equipotent in antimicrobial activity to PD-L4 and elicited an immune response from human cells (Pizzo et al. 2015). The PD-L1/2-derived peptides IKY31 and IKY23 exerted activity against planktonic bacterial cells as well as bacterial biofilms. They displayed stable helical conformations when exposed to membrane-mimicking agents and beta structures when exposed to components of the bacterial cell wall. RIPs may serve as precursors of antimicrobial and anti-biofilm agents, which are produced by proteolytic action to act against pathogens (Pizzo et al. 2018).

Flowering plant small type II RIP

Cinphorin

Cinphorin is a small type II RIP from seeds of the camphor tree (Cinnamomum camphora). Following reduction, both cinphorin and cinnamomin demonstrated RNA N-glycosidase activity and translation inhibitory activity. The molecular weight and first ten N-terminal amino acids of the A and B chains of cinphorin were identical to those of the type II RIP cinnamomin from the same seeds. The A chain of cinphorin possessed only half the molecular weight of the A chain of cinnamomin although the B chains of cinphorin and cinnamomin were of the same size. Cinphorin mRNA was undetectable by Northern blotting and RT-PCR. Cinphorin may be a protein splicing product of cinnamomin (Hou et al. 2002).

Small flowering plant RIPs

Two 11-kDa wax gourd (Benincasa hispida) seed peptides, designated as alpha-and beta-benincasins, both with an N-terminal sequence enriched in arginine and glutamine residues, were purified using affinity chromatography on Affi-gel blue gel, cation-exchange chromatography on Mono S, and gel filtration on Superdex 75. Alpha-benincasin inhibited mycelial growth in the fungi Physalospora piricola and Coprinus comatus but was devoid of suppressive activity on Mycosphaerella arachidicola. It inhibited protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 20 pM, more potently than beta-benincasin (IC50 = 320 pM) (Ng et al. 2003a, b).

Gamma-momorcharin

This 11.5-kDa small RIP from bitter gourd (Momordica charantia) seeds enriched in arginine and glutamate/glutamine at its N-terminal manifested translation inhibitory activity with an IC50 of 55 nM in the cell-free rabbit reticulocyte lysate system. Gamma-momorcharin acted on adenosine at position 4324 in a highly conserved loop of 28S ribosomal RNA (Pu et al. 1996).

Charantin

The 9.7-kDa charantin from bitter gourd seeds, which manifested pronounced N-terminal sequence homology to the 7.8-kDa bitter gourd napin-like peptide, inhibited cell-free protein synthesis in the rabbit reticulocyte lysate system with a higher IC50 (400 nM) than that of gamma-momorcharin (55 nM). Results of the N-glycosidase assay revealed the presence of charantin resulted in formation of a band similar to that formed by the small RIPs gamma-momorcharin and luffin S (Parkash et al. 2002a, b).

Hispin

A 21-kDa RIP designated as hispin, manifesting N-glycosidase and antifungal activities and a novel N-terminal sequence, was isolated from hairy melon (Benincasa hispida var. chieh-gua) seeds. It exhibited cell-free translation inhibitory activity in the cell-free rabbit reticulocyte lysate system with an IC50 of 165 pM. The isolation protocol entailed affinity chromatography on Affi-gel blue gel and cation exchange chromatography on CM-Sepharose and Mono S (Ng and Parkash 2002).

Lagenin

The 20-kDa lagenin from bottle gourd (Lagenaria siceraria) seeds reduced cell-free protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 0.21 nM and catalyzed hydrolysis of yeast tRNA with an activity of 45 U/mg. Its N-terminal sequence showed differences from counterparts in Family Cucurbitaceae, including elimination of the first three residues and a substitution of the 4th (Phe), 17th (Phe), 18th (Ile), and 22nd (Arg) amino acids invariant in other RIPs. The protein was unbound on DEAE-cellulose, but bound on Affi-gel blue gel as well as CM-Sepharose CL-6B (Wang and Ng 2000a, b).

Luffin-S

This small RIP from sponge gourd (Luffa cylindrica) seeds had a molecular weight of about 10 kDa, much smaller than those of the homologous RIPS luffin-a (27 kDa) and luffin-b (28 kDa). It displayed an alpha-sarcin-like mechanism on ribosomal RNA and translation inhibitory activity analogous to trichosanthin (Gao et al. 1994).

Luffacylin

The 7.8-kDa luffacylin from sponge gourd seeds was an RNA N-glycosidase that suppressed protein synthesis in the cell-free rabbit reticulocyte lysate with an IC50 of 140 pM. Its N-terminal sequence showed pronounced homology to that of the 6.5-kDa sponge gourd arginine-glutamate rich seed polypeptide. Luffacylin impeded mycelial growth in M. arachidicola and Fusarium oxysporum (Parkash et al. 2002a).

Luffin P1

The 5226-Da luffin P1 exhibited RNA N-glycosidase activity and hence suppressed protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 0.88 nM. Conversion of luffin P1 to a polymer enhanced its rRNA N-glycosidase activity (Li et al. 2003a, b, c). Luffin P1 demonstrated a mechanism dissimilar from the N-glycosidase activity of other RIPs. It is probably associated with charge complementation with viral or cellular proteins (Ng et al. 2011).

The isolation procedure of luffin P1 involved ammonium sulfate precipitation, cation exchange chromatography on CM-cellulose 52, affinity chromatography on Blue-gel and FPLC-cation exchange chromatography on Mono S. The sequence of the first eleven N-terminal amino acids was the same as the N-terminal sequence (from G3 to R13) of 6.5-kDa Arg/Glu-rich Luffa cylindrica seed peptide. Luffin P1 also showed pronounced sequence resemblance to pumpkin seed trypsin inhibitor C2 peptide. Luffin P1 suppressed trypsin activity and translation in the cell-free rabbit reticulocyte lysate system with an IC50 of 22 μM and 0.6 nM, respectively (Li et al. 2003a, b, c).

Recombinant human interleukin-2-luffin P1 (hIL-2-luffin P1) immunotoxin suppressed T cell proliferation in mixed lymphocyte reaction and response to the mitogen Concanavalin A with an IC50 of 1.8–10 nM. hIL-2-luffin P1 extended the survival time of animals bearing major histocompatibility complex-mismatched skin and kidney allografts (Wang et al. 2010).

Luffin P1 was fused to the C-terminal of an antibody against epidermal growth factor receptor (EGFR). The recombinant protein was expressed in E. coli, refolded and fractionated using immobilized nickel-affinity chromatography. The protein demonstrated specific binding and pronounced cytotoxicity to EGFR-positive cancer cells such as U251 cells, but manifested virtually no binding to EGFR-negative cells such as Jurkat cells (Ma et al. 2012).

Luffangulin

The 5.6-kDa luffangulin from Luffa acutangula seeds, with an arginine/glutamate-rich N-terminal sequence, suppressed protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 3.5 nM, but it was devoid of HIV-1 reverse transcriptase inhibitory activity (Wang and Ng 2002).

Cucurmoschin

The 8-kDa peptide from black pumpkin seeds, with an abundance of arginine, glycine, and glutamate at the N-terminal, manifested antifungal activity toward F. oxysporum, Botrytis cinerea, and M. oxysporum. The peptide suppressed protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 1.2 μM. Like the vast majority of RIPs, it was bound on Affi-gel blue gel but not on DEAE-cellulose (Wang and Ng 2003).

Moschins

From fresh brown pumpkin (Cucurbita moschata) seeds, two 12-kDa proteins designated alpha-moschin and beta-moschin characterized by an N-terminal sequence containing an abundance of arginine and glutamate residues were reported. The N-terminal sequences of alpha- and beta-moschins displayed pronounced homology to Drosophila apoptosis gene product and prepro 2S albumin, respectively. Alpha- and beta-moschins exhibited cell-free translation inhibitory activity in the cell-free rabbit reticulocyte lysate system with an IC50 of 17 μM and 300 nM, respectively (Ng et al. 2002).

Pisavins

Alpha- and beta-pisavins, two ribosome-inactivating proteins designated from garden pea (Pisum sativum var. arvense Poir) seeds with N-terminal amino acid sequence identity, differed in molecular weight, 20.5 kDa and 18.7 kDa, respectively. Merging of the two proteins into a single band with an isoelectric point exceeding 9.3 was observed in isoelectric focusing. Both suppressed cell-free translation in the rabbit reticulocyte lysate system with an IC50 close to 0.5 nM and displayed RNase activity toward transfer RNA and N-glycosidase activity toward ribosomal RNA to produce an Endo's fragment. Circular DNA and supercoiled DNA were transformed, in the presence of the pisavins, into a linear form (Lam et al. 1998).

S-trichokirin

The 8-kDa strongly basic RNA N-glycosidase from Trichosanthes kirilowii seeds, designated as S-trichokirin which is a basic protein with a pI near pH 9, demonstrated RNA N-glycosidase activity and suppressed protein biosynthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 0.115 nM. Its isolation procedure involved ammonium sulfate precipitation, cation exchange chromatography on CM-52, gel filtration on Sephacryl S-100 and FPLC-cation exchange chromatography on Mono S (Tai et al. 2000).

Trichokirin-S1

Trichokirin-S1, an 11.426-kDa rRNA N-glycosidase from T. kirilowii seeds, manifested translation inhibitory activity in the cell-free rabbit reticulocyte lysate system with an IC50 of 0.7 nM (Li et al. 2003a, b, c).

Trichosanthrip

Trichosanthrip from mature T. kirilowii seeds, which exhibited a molecular weight of 10.965-kDa, impeded protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of about 0.15 nM and demonstrated N-glycosidase activity toward 28 S rRNA (Shu et al. 2009).

Mushroom type II RIP

Polyporus umbellatus type II RIP

A gene cloned from P. umbellatus sclerotia by RT-PCR encoded a 32.33-kDa type IIRIP with an isoelectric point of 5.58. The RIP contained conserved domains of RICIN superfamily protein. It bore a relationship to Marasmius oreades RIP. The gene of P. umbellatus RIP was expressed in a variety of tissues of the mushroom examined. Upregulation of the gene was observed in the Armillaria mellea–infected parts of the mushroom (Liu et al. 2017).

Mushroom RIPs

Calcaelin

Calcaelin from fresh fruiting bodies of the mosaic puffball mushroom Calvatia caelata was bound on Affi-gel blue gel and unbound on DEAE-cellulose. It was a 39-kDa protein composed of a 19-kDa subunit and a 20-kDa subunit. The two subunits were identical in N-terminal amino acid sequence which partially resembled plant RIPs and exhibited weaker similarity to the fungal ribosome-inactivating proteins restrictocin and alpha-sarcin. Calcaelin suppressed protein synthesis in the cell-free rabbit reticulocyte lysate with an IC50 value of 4 nM. Calcaelin exhibited antiproliferative activity toward breast cancer cells, antimitogenic activity toward mouse splenocytes, and RNase activity of 1.58 U/mg toward yeast tRNA (Ng et al. 2003a, b).

Flammulin, flammin, velin, and velutin

Flammulin from fruiting bodies of the golden needle mushroom Flammulina velutipes displayed a molecular weight of 40 kDa, and an N-terminal sequence analogous to those of plant RIPs. The protocol for its purification involved anion exchange chromatography on DEAE-cellulose, cation exchange chromatography on SP-Sepharose, and affinity chromatography on Affi-gel

blue gel. Flammulin was unbound on the weak anion exchanger DEAE-cellulose at neutral pH and low ionic strength but bound on the weak cation exchanger CM-Sepharose and the affinity chromatographic media Affi-gel blue gel under similar conditions. Flammulin suppressed protein synthesis in a cell-free rabbit reticulocyte lysate system with an IC50 of 0.25 nM (Wang and Ng 2000a, b).

Velutin from F. velutipes possessed a low molecular weight of 13.8 kDa and an N-terminal sequence with some resemblance to those of plant RIPs. The protocol for its purification involved chromatography on the anion exchanger DEAE-cellulose, chromatography on the strong cation exchanger SP-Sepharose, and finally affinity chromatography on Affi-gel blue gel. Velutin exhibited inhibitory activity toward human immunodeficiency virus type 1(HIV-1) reverse transcriptase, beta-glucosidase, and beta-glucuronidase (Wang and Ng 2001a, b). Glucosidases facilitate HIV gp120 glycosylation. Glycohydrolase inhibition reduces HIV infectivity (Collins and Ng 1997; Collins et al. 1997).

Flammin and velin from F. velutipes fruiting bodies displayed a molecular mass of 30 kDa and 19 kDa, respectively. Although both of them were unbound on DEAE-cellulose and bound on Affi-gel blue gel and CM-Sepharose, they could be separated by fast protein liquid chromatography-gel filtration on Superdex 75. Flammin exhibited only slight N-terminal sequence homology to angiosperm type 1 RIPs such as alpha-momorcharin, beta-momorcharin, and trichosanthin, but did not resemble other mushroom RIPs. Velin showed some similarity to the A chain of abrin, a type 2 RIP. Flammin and velin repressed protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 1.4 nM and 2.5 nM, respectively. Neither of them exhibited protease or ribonuclease activity (Ng and Wang 2004).

Hypsin

Hypsin is a 20-kDa RIP from fruiting bodies of the mushroom Hypsizigus marmoreus. The protocol for its purification involved cation exchange chromatography on CM-cellulose, affinity chromatography on Affi-gel Blue Gel, and finally chromatography on the strong anion exchanger Mono Q. It inhibited protein synthesis in cell-free rabbit reticulocyte lysate, HIV-1 reverse transcriptase activity, and thymidine uptake by murine splenocytes with an IC50 of 7 nM, 7 nM, and 1 µM, respectively. Hypsin exhibited antifungal activity toward B. cinerea, F. oxysporum, M. arachidicola, and P. piricola, with an IC50 of 0.06, 14.2, 2.7, and 2.5 µM, respectively. It inhibited protein synthesis in cell-free rabbit reticulocyte lysate and HIV-1 reverse transcriptase activity, with an IC50 of 7 nM and 8 µM, respectively. The protein synthesis inhibitory activity was relatively thermostable and trypsin-stable as approximately 60% of the inhibitory activity remained following exposure to the boiling temperature for 10 min and 100% activity was recovered after trypsin treatment for a short duration. Hypsin inhibited proliferation of human leukemia and hepatoma cells and mouse leukemia cells (Lam and Ng 2001a, b).

Hypsin from H. mamoreus at 2.5 µM elicited a dose-dependent deranged development in cultured murine embryos during the organogenesis period from E8.5 to E9.5. A reduction in the final somite number, and an increase in structural abnormalities such as a patent cranial neural tube, aberrant branchial arches, lack of forelimb buds and a twisted body axis, were observed. Nevertheless, the otic and optic placodes were affected to a lesser extent. Hypsin

also cause more cell death. In stark contrast, velutin did not have any teratogenic effects on mouse embryos (Ng et al. 2010).

Marmorin

Marmorin from fresh fruiting bodies of the mushroom H. marmoreus displayed a novel N-terminal sequence and a molecular weight of 9.567 kDa. It was purified by ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, and gel filtration on Superdex 75. Marmorin was not bound on DEAE-cellulose but was bound on Affi-gel blue gel. Marmorin exhibited antiproliferative activity toward breast cancer MCF-7 cells with an IC50 of 5 µM, hepatoma Hep G2 cells with an IC50 of 0.15 µM, RIPs from bitter gourd, garden pea, hairy gourd, ridge gourd, and the mushroom F. velutipes. Marmorin inhibited protein synthesis in the cell-free rabbit reticulocyte lysate system and HIV-1 reverse transcriptase activity with an IC50 of 0.7 nM and 30 µM, respectively. Marmorin was lacking in RNase, protease, trypsin inhibitory, hemagglutinating, mitogenic, anti-mitogenic, nitric oxide-inducing, and antifungal activities (Wong et al. 2008).

Marmorin reduced the survival of breast cancer in vitro and in vivo. Estrogen receptor (ER)–positive MCF7 breast cancer cells were more responsive than ER-negative MDA-MB-231 cells to marmorin. Estrogen receptor α (ERα) expression was attenuated, and 17β-estradiol-induced proliferation of MCF7 cells was suppressed by marmorin. ERα knockdown in MCF7 cells led to a decrement in the antiproliferative potency of marmorin, signifying the involvement of the ERα-mediated pathway in the activity of marmorin on ER-positive breast cancer cells. Apoptoses in both MCF7 and MDA-MB-231 cells were brought about by marmorin in a time-dependent manner. Activation of caspase-9 in the breast cancer cells, depolarization of their mitochondrial membrane potential, and arrest of the breast cancer cells in the G2/M-phase, took place in MCF7 cells and to a less pronounced way in MDA-MB-231 cells in response to marmorin. In addition, in both MCF7 and MDA-MB-231 cells, marmorin activated the death receptor apoptotic pathway (through caspase-8 activation) and endoplasmic reticulum stress (through PERK and IRE1α phosphorylation, caspase-12 cleavage and CHOP expression upregulation).

Lyophyllin

Lyophyllin from fruiting bodies of the mushroom Lyophyllum shimeji possessed a molecular weight of 20 kDa and an N-terminal sequence resembling those of angiosperm RIPs. It exerted an antifungal action against C. comatus and P. piricola (IC50 = 2.5 µM). Lyophyllin and the antifungal protein from L. shimeji synergized in their action against the fungus P. piricola. The protocol for its purification involved cation exchange chromatography on CM-cellulose, affinity chromatography on Affi-gel Blue Gel, and finally cation exchange chromatography on Mono S. It inhibited protein synthesis in the cell-free rabbit reticulocyte lysate, HIV-1 reverse transcriptase activity, and thymidine uptake by murine splenocytes with an IC50 of 1 nM, 7.9 nM, and 1 µM, respectively. Lyophyllin did not have hemagglutinating or ribonuclease activity (Lam and Ng 2001a, b).

Lyophyllin microinjected at a concentration as low as 50 µg/ml into cultured postimplantation mouse embryos during the period of organogenesis from E8.5 to E9.5 produced dose-

dependent abnormalities. The affected embryonic structures adversely affected by lyophyllin comprised the body axis, the final somite number, cranial neural tube, branchial arches, and forelimb buds. The otic and optic placodes were more recaltrant. Forebrain blisters within the cranial mesenchyme were observed at lyophyllin doses above 500 µg/ml. Histologically observed cell death rose concurrently with the increased appearance of abnormal structures (Chan et al. 2010).

Pleuturegin

Pleuturegin from fresh sclerotia of the mushroom Pleurotus tuber-regium differed from the bulk of plant and mushroom RIPs in its adsorption on DEAE-cellulose and unadsorption on SP-Sepharose, but like other RIPs it was adsorbed on Affi-gel blue gel. Its N-terminal sequence did not share similarity with other mushroom RIPs including flammulin, hypsin, lyophyllin, and velutin. It had a larger molecular weight (38 kDa) than those of hypsin and lyophyllin (both 20 kDa) and velutin (13.8 kDa) but resembled flammulin (40 kDa) in molecular weight. Pleuturegin was lacking in ribonuclease activity. It suppressed protein synthesis in a cell-free rabbit reticulocyte lysate system with an IC50 of 0.5 nM (Wang and Ng 2001a, b).

Bacterial RIPs

Shiga toxin

Shiga toxins are bacterial proteins produced by Shigella dysenteriae and some E. coli strains. They are bacterial type 2 RIPs with an AB5 structure composed of a catalytic N-glycosidase A chain (which depurinates a universally conserved adenine residue in the α-sarcin/ricin loop of the 28S ribosomal RNA) and five identical B chains and bind to the target cells with specific sugars. Gb3 receptors on the brain, intestinal, and renal cells recognize Shiga toxins and are damaged by the toxins (Chan and Ng 2016). Severe Shiga toxin intoxication results in hemolytic-uremic syndrome, which may lead to renal failure and death. Production of recombinant Shiga toxin from non-toxic recombinant Shiga toxin A subunit and recombinant Shiga toxin B subunits allows screening and assessment of inhibitors of a small size for development of therapeutic agents (Chauhan et al. 2019). Shiga toxin producing E. coli O157:H7 causes food-poisoning. Some of these strains secrete Shiga toxin 1 and/or Shiga toxin 2 or variants which cause hemolytic uremic syndrome or hemorrhagic colitis. The strains producing Shiga toxin 2 have greater virulence and connection with hemolytic-uremic syndrome. Shiga toxins 1 and 2 exhibit differences in structure, receptor binding, dependence on ribosomal proteins, and pathogenicity. The B subunit and perhaps the A1 subunit as well may contribute to the dissimilar toxicities of Shiga toxins 1 and 2 (Basu and Tumer 2015).

Shiga toxins and the toxic plant type II RIP ricin trigger apoptotic cell death in myeloid, lymphoid endothelial and epithelial cells in vitro, and in a number of organs. There may be dissociation between apoptosis and suppression of protein synthesis brought about by ricin and Shiga toxins. The B chain of ricin and B subunits of Shiga toxin upregulate both the death receptor-mediated (extrinsic) and mitochondrial-mediated (intrinsic) pathways of apoptosis induction in some types of cells (Tesh 2012a, b).

Shiga toxins upregulate different pathways connected with stress. Subsequent to depurination in the α-sarcin/ricin loop of eukaryotic ribosomes, the ribotoxic stress response and thus the mitogen-activated protein kinase (MAPK) cascade is triggered signaling through MAPK cascades, which in turn activates innate immunity and controls apoptosis. Shiga toxin unfolding within the endoplasmic reticulum may initiate the unfolded protein response which is continued by formation of truncated, misfolded proteins. Treatment to prevent damage produced by Shiga toxin and progression of disease may be facilitated by targeting sites on the stress response pathways (Tesh 2012a, b).

Streptomyces coelicolor RIP

RIP-like proteins similar to plant type I RIPs rather than the RIP subunit (Shiga toxin A) of Shiga toxin were found in the genomes of two actinomycete bacteria. RIP-like proteins similar to Shiga toxin A were noted in the genomes of β- and γ-protebacteria The 30-kDa RIP-like gene product SCO7092 from the Gram-positive soil bacterium Streptomyces coelicolor (RIPsc) was an N-glycosidase. Excessive formation of RIPsc in E. coli and Streptomyces lividans seriously retarded growth. S. coelicolor RIP suppressed protein synthesis in a cell-free system but failed to inhibit fungal and bacterial cells owing to inability to enter the cells (Reyes et al. 2010).

Spiroplasma RIP

The fruitfly Drosophila neotestacea is protected against infection caused by the nematode parasite Howardula aoronymphium with the assistance of its symbiotic bacterium Spiroplasma. Spiroplasma produces an RIP similar to E. coli Shiga-like toxins and depurinates H. aoronymphium ribosomal RNA. Recombinant Spiroplasma RIP catalyzes the cleavage of purine from 28S rRNA and also from Howardula rRNA at the target site of RIPs within the α-sarcin/ricin loop of 28S rRNA without affecting fruitfly 28S rRNA (Hamilton et al. 2016).

Out of the several RIP genes in the genome of Spiroplasma poulsonii, SpRIP1 and SpRIP2 are the only RIPs expressed throughout the life cycle of the fruitfly. SpRIP1 and 2 expression in uninfected flies brought about a shortening of fruitfly lifespan and hemocyte count and increase embryonic mortality (Garcia-Arraez et al. 2019).

Various Spiroplasma strains, which comprise the male-killing symbiont (sMel) of D. melanogaster, are protective against parasitic wasps, but only the sNeo strain which produces infections in D. neotestacea is protective against nematode parasites. The RIP determines whether there is protective activity against nematodes. RIPs of the sMel Spiroplasma strain are devoid of inhibitory activity toward nematode ribosomes in vivo (Ballinger et al. 2019). A Spiroplasma strain which infects a mycophagous phorid fly Megaselia nigra was found. The M. nigra symbiont codes for different RIPs which comprise plasmid-encoded toxins with close relationship to sNeo RIPs (Ballinger et al. 2019).

Mateos et al. (2019) conducted a study to ascertain whether inheritable endosymbionts which gave rise to different D. melanogaster reproductive phenotypes affected the mRNA transcriptome of early embryos. mRNA-seq was employed to assess differential expression in

Drosophila embryos devoid of endosymbionts (control) in comparison with those containing S. poulsonii strain Hyd1 which is a strain naturally associated with Drosophila hydei and devoid of a reproductive phenotype; the male-killing S. poulsonii strain MSRO-Br; and the cytoplasmic incompatibility–inducing Wolbachia strain wMel. No effect of the symbiont was detected on mRNA composition of early embryos, indicating that the mechanism of reproductive manipulation does not entail changes of maternally loaded transcripts. mRNA-seq datasets from D.melanogaster embryos infected with Spiroplasma were used to look for rRNA depurination signals. Signals of rRNA depurination with slightly greater intensity were detected in treatment with male-killing S. poulsonii strain MSRO-Br than those from treatment with S. poulsonii strain Hyd1, but signals were not detected in the symbiont-free control or treatment with the cytoplasmic incompatibility–inducing Wolbachia strain wMel, in keeping with the RIP action and mortality of male embryos caused by RIP (Mateos et al. 2019).

Phylogenetic distribution of RIPs

RIPs show a wide distribution in plants and in a few bacterial, fungal and metazoan species, suggesting an ancestral RIP domain in plants subsequently acquired by bacteria via horizontal gene transfer. This observation, together with sequence and phylogenetic analyses, prompted Lapadula et al. (2013) to put forward a hypothesis of the origin and evolution of the RIP domain, which suggested existence of several paralogous RIP genes prior to evolution of the three domains of life, in keeping with the contemporary concept of the Last Universal Common Ancestor (LUCA) as a complex, genetically redundant organism. Differential loss of paralogs in LUCA descendants, instead of multiple horizontal gene transfer events, may explain the complex pattern of RIP genes across existing species, as seen in other genes.

Activation of RIPs

The mechanisms involved in the activation of cereal RIPs comprise cleavage of internal peptides from the N-glycosidase domains of the RIPs by proteases (Bass et al. 2004; De Zaeytijd and Van Damme 2017). Reductive cleavage of the RIP A-chain of type II RIPs like ricin from the cell-binding B chain, catalyzed by oxidoreductases such as protein disulfide isomerase and TMX (a transmembrane thioredoxin-related protein in the protein disulfide isomerase family) in the endoplasmic reticulum lumen, plays a role in the activation of ricin. DU145 prostatic cells in which TMX has been silenced exhibited diminished susceptibility to ricin. In contrast, exposure of lung cancer A549 cells control cells to Pseudomonas exotoxin A or saporin whose activation mechanism is only partly related and unrelated to reduction, respectively, did not lead to differences in cytotoxicity (Pasetto et al. 2012).

Entry to cells

Ricin dimers gain entry to target mammalian cells through receptor-mediated endocytosis. Saporin shows binding in vitro to alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein (LRP1) which mediates saporin internalization in human monocytes and in fibroblasts (De Virgilio et al. 2010).

Activity of RIPs toward RNA

Treatment of eukaryotic ribosomes with plant RIPs proteins sensitized the 28S rRNA of eukaryotic ribosomes to aniline treatment, resulting in breakage of the N-glycosidic bond at A-4324 in the highly conserved sarcin/ricin domain loop of 28S rRNA and production of an RNA fragment consisting of approximately 450 nucleotides known as the Endo's band (Endo et al. 1988). The type I RIP trichosanthin and type II RIP cinnamomin produced the Endo's band and impeded protein synthesis (Liu et al. 2017). On the other hand, B. orientalis RNase broke a specific phosphodiester bond between C4453 and A4454 of 28SRNA and generated a small RNA-fragment (S-fragment) hence halting protein synthesis. The kelp RIP lamjapin also hydrolyzed 28S RNA to produce an RNA fragment smaller than the diagnostic R-fragment (Liu et al. 2017).

Settings

Assays of activity of RIPS

Advantages and the disadvantages of the assays of activity of RIPS including depurination assays (aniline cleavage assay, primer extension assay, qRT-PCR assay, adenine detection assay, enzymatically coupled adenine detection assay) and translation inhibition assays (in vitro translation inhibition assay, in-cell radioactive amino acid incorporation assay, in-cell luciferase synthesis assay, cell-based luciferase assay, in-cell GFP synthesis assay) have been evaluated by Zhou et al. (2018).

Conclusion

Single-chained type I RIPs may be found together with double-chained type II RIPs in the same plant (de Benito et al. 1995; Fong et al. 1996; Ling and Liu 1996; Fang et al. 2012). It is noted that small RIPs may coexist with regular-sized RIPs in the same plant, such as alpha-momorcharin, beta-momorcharin, gamma momorcharin, and charantin in bitter gourd (M. charantia) seeds; alpha-and beta-kirilowins (Dong et al. 1994; Wong et al. 1996), S-trichokirin (Tai et al. 2000), trichokirin-S1 (Li et al. 2003a, b, c), and trichosanthrip (Shu et al. 2009) in T. kirilowii seeds; luffin-a, luffin-b, luffin-S, luffin P, and luffacylin in sponge gourd (L. cylindrica) seeds; luffaculin and luffangulin in ridge gourd (L.acutangula) seeds; and C. moschata RIP, cucurmoschin and moschins in pumpkin (C. moschata) seeds. Nevertheless, the small RIPs hispidin, lagenin, benincasins, and pisavins are found in the absence of regular RIPs in seeds of hairy gourd (B. hispida var. chieh qua), bottle gourd (L. siceraria), wax gourd (B. hispida), and garden pea (P. sativum). Only regular-sized RIPs are found in some seeds. Overall speaking, issues of some plant show presence of both regular-sized (RIPs and small RIPs, while other plants show presence of only small RIPs or only regular-sized RIPs (Tables 1 and 2).

Table 1 RIPs covered in this review with their molecular weights and IC50s in translation in rabbit reticulocyte lysate

Full size table

Table 2 Presence of regular-sized ribosome-inactivating proteins (RIPs) alone, small RIPs alone, and concurrent existence in plant tissues

Full size table

As judged from the complete amino acid sequences of the regular-sized RIPs and the partial sequences of the homologous small RIPs, the latter RIPs are not derived from the former RIPs. Chromatographic behavior of the vast majority of regular-sized RIPs resembles that of small RIPs in adsorption on cationic exchangers such as CM-cellulose/Mono S and the affinity chromatographic media Blue-Sepharose or Affi-gel blue gel and non-adsorption on anionic exchangers such as DEAE-cellulose (Ng et al. 1992; Fong et al. 1996; Wang and Ng Wang and Ng 2000a, b, 2001a, b, Wang et al. 2012; Li et al. 2003a, b, c). Small plant RIPs can be readily separated from the regular-sized counterparts by chromatography (Wang and Ng 2003).

RIPs manifest an array of molecular weights and amino acid sequences. It is known that proteins with similar functions may differ appreciably in structural characteristics such as molecular weight and amino acid sequence. This is amply exemplified by milk proteins and derived peptides; antifungal, antibacterial, and antiviral proteins/peptides produced by different organisms; and animal and plant hemagglutinins/lectins. Furthermore, different mechanisms may be employed to elicit the same activity (Wong et al. 2010; Cheung et al. 2014;Ng et al. 2015, 2016; Yan et al. 2015; Lam and Ng 2011; Lönnerdal et al. 2017; Shafee et al. 2017; Sierra et al. 2017; Neshani et al. 2019;Wong et al. 2019). Plant RIPs recognize a universally conserved α-sarcin/ricin loop in rRNA and remove a single adenine (A4324 in 28S rat rRNA), thereby halting protein synthesis and eliciting death in mammalian cells (Fabbrini et al. 2017). Small plant RIPs have also demonstrated similar N-glycosidase activity (Pu et al. 1996; Lam et al. 1998; Tai et al. 2000; Parkash et al. 2002a, b; Li et al. 2003a, b, c; Shu et al. 2009). Spiroplasma RIP catalyzes the splitting of purine from 28S rRNA and also from Howardula rRNA at the target site of RIPs within the α-sarcin/ricin loop of 28S rRNA without affecting fruitfly 28S rRNA (Hamilton et al. 2016). The catalytic N-glycosidase A chain of Shiga toxins also eliminates a conserved adenine residue in the α-sarcin/ricin loop of the 28S ribosomal RNA (Chan and Ng 2016). The 13-kDa B. orientalis RNase (RNase Bo) cleaves a phosphodiester linkage located between C4453 and A4454 referred to as B. orientalis RNase region of 28S RNA in rat ribosomes in the presence of 25 mM magnesium ions. A small RNA-fragment designated as the S-fragment is generated and this action resembles that of α-sarcin. The cleavage-site of α-sarcin (G4325) is separated by 128 nucleotides from that of B. orientalis RNase (C4453). The damaged ribosome is incapable of binding aminoacyl-transfer RNA (Xu et al. 2004; Liu et al. 2017).

RIPs have HIV enzyme inhibitory, antiviral, antifungal, antibacterial, anti-insect, antiprotozoal, and anticancer activities (Wong et al. 2010; Wang et al. 2012; Akkouh et al. 2015; Bolognesi et al. 2016; Ng et al. 1992, 2015, 2016; Zhu et al. 2018; Alves de Vasconcelos et al. 2018) and have potential applications in medicine and agriculture. Plant RIPs act against a variety of viruses including human immunodeficiency virus, hepatitis B virus, and herpes simplex virus. A mechanism of antiviral activity may involve inactivation of host ribosome, inhibition of viral protein synthesis and host cell death. (Kaur et al. 2011). Regular-sized RIPs express antiproliferative activity toward cancer cells and anticancer activity toward tumor bearing mice (Zeng et al. 2015). RIPs trigger apoptosis in cancer cells through mitochondrial, death receptor, and endoplasmic reticulum pathways (Zeng et al. 2015). Small RIPs from plants also demonstrate antifungal (Wang and Ng 2001a, b; Ng et al. 2002; Parkash et al. 2002a, b; Ng et al. 2003a, b), and HIV-I reverse transcriptase inhibitory activity (Wang and Ng 2003). Small RIPs from mushrooms display antifungal, anticancer, and HIV-I reverse transcriptase inhibitory activities (Lam and Ng 2001a, b; Wang and Ng 2001a, b; Wong et al. 2008; Pan et al. 2013).

Fungal ribotoxins have anti-insect (Dowd et al. 2012; Chuang et al. 2014) and anti-HIV activities (Yadav and Batra 2015).

Immunotoxins based on RIPs have anticancer activity (Polito et al. 2011). Molecular cloning of RIPs, expression and purification of recombinant RIP have been reported (Wang et al. 2012). Immunotoxins have also reported based on Sambucus type 2 RIPs which are nontoxic because of an intracellular pathway not leading to contact with ribosomes (Ferreras et al. 2011). Methods have been proposed to enhance the clinical usefulness of saporin-based immunotoxins including combinatorial strategies to promote RIP escape into the cytoplasm and genetic manipulations to reduce undesirable events like vascular-leak syndrome or identify T/B cell epitopes to mitigate immunogenicity (Giansanti et al. 2018). Many eminent scientists have contributed to the voluminous literature on RIPs, one of whom is Professor Fiorenzo Stirpe (Bolognesi et al. 2017). Immunotoxins based on small RIPs like trichokirin-S have been proposed (Li et al. 2003a, b, c).

The mushroom RIPs lyophyllin and hypsin displayed teratogenic effects on cultured mouse embryos. Straw mushroom RIP volvarin exhibited abortifacient activity in pregnant mice. The findings are in keeping with observations on regular RIPs including momorcharins, trichosanthin, and luffins. However, the small plant RIPs have not been so tested.

Only recently have RIPs been found in invertebrate animals (Lapadula et al. 2017). To date no vertebrate RIPs have been reported. In view of the myriad of invertebrate and vertebrate species in the animal kingdom, it is likely that many other animal RIPs remain to be uncovered. Delving into the roles of animal RIPs is undoubtedly a worthwhile undertaking.

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