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Mini-review

Ribosome-inactivating proteins: From toxins to useful proteins

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ABSTRACT

Ribosome-inactivating proteins (RIPs) either single-chain (type 1) or two-chain (type 2) are frequent in plants, often in multiple forms. They are RNA *N*-glycosidases, have antiviral, antifungal and insecticidal activity. Their expression in plants is increased under stressful conditions. They are investigated for practical applications in medicine and in agriculture. In medicine, RIPs have been linked to, or fused with, appropriate antibodies or other carriers to form "immunotoxins" or other conjugates specifically toxic to the cells target of the carrier, with the aim of eliminating malignant or other undesired cells. In agriculture, it has been observed that an enhanced expression of RIPs confers to plants an increased resistance to viruses, fungi, insects, and also to drought and salinity.

1. Introduction

Ribosome-inactivating proteins (RIPs) are a family of proteins that have been identified and described in the last forty years. Fairly soon several possible applications of these proteins were envisaged, some of which appear close to realization.

Ribosome-inactivating proteins have been found in plants, mushrooms and bacteria, often in multiple forms and have been classified as type 1, consisting of a single chain with enzymatic activity, and type 2, consisting of an A-chain similar to type 1 RIPs, linked to a B-chain with properties of a lectin specific for sugars with the galactose structure (reviews by Van Damme et al., 2001; Girbés et al., 2004; Stirpe and Battelli, 2006; Puri et al., 2012). A type 3 RIP also has been described, consisting of a single chain with an additional protein segment that must be removed for the RIP to be active (Peumans et al., 2001). More type 1 than type 2 RIPs have been identified, and were detected in many plants, including several vegetables which are eaten raw (Barbieri et al., 2006). The hypothesis that they could be present in all plants should be dismissed, because a gene for them was not found in the genome of *Arabidopsis thaliana*. In many plants RIPs are found in several tissues (e.g. saporin in *Saponaria officinalis*), in other plants in one tissue only (e.g. ricin in the seeds of *Ricinus communis*).

The enzymatic activity of RIPs is an *N*-glycosidase (rRNA *N*-glycosidase, EC 3.2.22), which removes a single adenine from rRNA (A_{4324} from the 28S rRNA in the 60S subunit of rat liver ribosomes), thus causing inhibition of protein synthesis. RIPs also remove adenine from DNA and other polynucleotides, although with variable efficiency, and consequently the denomination of adenine polynucleotide glycosylase was proposed for these proteins (Barbieri et al., 2001). It has been reported that other enzymatic activities are associated with some RIPs: chitinase (Shih et al., 1997), superoxide dismutase (Li et al., 1997), DNase (Ruggiero et al., 2007), lipase (Lombard et al., 2001); however, the possibility that they result from contamination cannot be excluded.

The B-chains of type 2 RIPs are lectins which bind to galactosyl residues on the surface of animal cells, allowing

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the A-chains to enter the cytoplasm, where they damage ribosomes with consequent cell death (review by Sandvig and van Deurs, 2002). Thus some type 2 RIPs are potent toxins, ricin the best known, whereas others are not toxic, possibly due to differences in their entry into cells and intracellular routing and resistance to proteolysis (Battelli et al., 1997). Toxic type 2 RIPs have been used for homicidal purposes (Knight, 1979), and there are fears that ricin and related toxins could be used as biological weapons for warfare or terrorism (Griffiths, 2011).

Type 1 RIPs are much less toxic, because lacking the B-chain they do not bind to cells, in which they enter with difficulty, however, they can be toxic if they are conjugated to molecules capable of linking to cells.

Most RIPs have antiviral (Parikh and Tumer, 2004; Kaur et al., 2011), antifungal (Ng, 2004; Theis and Stahl, 2004), insecticidal (Carlini and Grossi-de-Sá, 2002) and abortifacient properties (Ng et al., 1992), and seem to have a role in plants under stressful conditions (see below). There have been attempts to exploit these properties of RIPs in medicine and in agriculture. Furthermore, several studies have been performed with RIPs linked to, or fused with, vector molecules capable of delivering them to target cells to be eliminated. These conjugates are research tools, particularly used in neurobiology research, (Wiley and Lappi, 2005), but are also studied for therapeutic uses (Fracasso et al., 2010).

This article will be focused on examples of practical applications of RIPs and their derivatives, which seem to be close to practical use.

2. Possible applications

2.1. In medicine

In medicine, the uses of unmodified type 1 RIPs are rather limited. Trichosanthin, a RIP from *Trichosanthes kirilowii*, is used in the official Chinese medicine to induce abortion and to treat hydatidiform moles (Ng et al., 1992). The inhibitory effect of RIPs on HIV proliferation in cells led to clinical trials on AIDS patients, unfortunately without success (reviews by Parikh and Tumer, 2004; Kaur et al., 2011).

Most research on the possible therapeutic use of RIPs was, and still is, focused on the possibility of directing them in a selective manner toward cells to be eliminated. This can be achieved by linking them to molecules, especially monoclonal antibodies, but also lectins, hormones, growth factors, to form "immunotoxins" or other cell-binding conjugates capable of specifically linking to unwanted cells (review in Fracasso et al., 2010). Whole type 2 RIPs cannot be used for this purpose, because their B chains would bind unspecifically to all sorts of cells, which would be killed. Thus separated A chains or type 1 RIPs were attached to antibodies or other carriers, initially by chemical linkages and subsequently by fusion technique, the latter giving the advantage of a constant composition of the resulting molecule.

Most studies were performed with the aim of eliminating malignant cells and many good results were obtained *in vitro* and in experimental animals (reviews by Frankel et al., 2000; Fracasso et al., 2010). Remissions were obtained also in some limited clinical trials (e.g. Falini et al., 1992) in most cases with controllable short-lasting side effects. Vascular leak syndrome and fatigue were particularly important and frequent (review by Litvak-Greenfeld and Benhar, 2012), and could be reduced by the use of mutated RIPs (e.g. ricin, Smallshaw et al., 2003). However, immunotoxins are foreign proteins and elicit an immunological response,which prevents repeated administration. This is the main obstacle to the use of immunotoxins, which could be circumvented in three ways.

The first approach is to reduce the immunogenicity of immunotoxins with the use of human or humanized antibodies and of RIPs modified by pegylation (e.g. Meng et al., 2012) or by depletion of immunodominant epitopes (Lorberboum-Galski, 2011). An immunotoxin prepared with a modified recombinant bouganin, a RIP from *Bougainvillea spectabilis* (Cizeau et al., 2009), caused scarce formation of antibodies when given to animals (Entwistle et al., 2012) and patients (Cizeau et al., 2012). A limited immune response was observed in patients receiving an immunotoxin constructed with carbohydrate-free recombinant gelonin, a RIP from *Gelonium multiforum* (Borthakur et al., 2013).

A second possibility is to administer immunotoxins in a district "external" to the immune system: patients with bladder cancer were treated with intravesical irrigation with immunotoxins constructed with ricin (Zang et al., 2000) or Pseudomonas exotoxin (Kowalski et al., 2012), with results reportedly comparable to, if not better than, those obtained with local chemotherapy.

Thirdly, immunotoxins can be efficiently used if a single administration is sufficient to achieve the desired effect before the immune reaction is mounted. Studies were performed with RIP conjugates aimed to suppress some forms of strong chronic pain through the permanent removal of a small number of spinal neurons which transmit chronic pain signals, without affecting the sensitivity to acute pain. Good results were obtained in experimental animals with saporin, a RIP from *S. officinalis*, linked to the pain-processing peptide Substance P (Mantyh et al., 1997).

In rats, a single administration of this conjugate is sufficient to eliminate a small number of cells that transmit chronic pain signals, causing a relief that appears to be permanent, whilst normal acute pain is unaffected. Promising results were obtained also with the use of saporin linked to the galactose-specific isolectin B4 from *Bandeiraea simplicifolia*, which eliminated chronic muscle pain in the rat (Alvarez et al., 2012).

2.2. In agriculture

In agriculture, research was performed to exploit mainly the antiviral, antifungal and insecticidal properties of RIPs. DNA recombinant technology was applied in plants to increase their resistance to various agents either by introducing a RIP gene derived from another plant or by manipulating the levels of their endogenous RIP. A complete review is beyond the aim of this present article, and only representative examples will be given.

An antiviral protein from *Phytolacca americana* (PAP) was the first purified RIP (Obrig et al., 1973). Both type 1 and type 2 RIPs were studied as antiviral agents (reviews by Wang and Tumer, 2000; Kaur et al., 2011). Improved resistance to viruses was obtained in several cases, e.g. in tobacco (Lodge et al., 1993) and bentgrass (Dai et al., 2003) plants transfected with pokeweed antiviral protein (PAP), and in tobacco plants transfected with the JIP60 RIP from barley (Görschen et al., 1997). Non-toxic Type 2 RIPs were also investigated, and antiviral protection was observed in transgenic tobacco plants transfected with SNA-I′ from *Sambucus nigra* (Chen et al., 2002), whereas a type 2 RIP from Iris gave local, but not systemic protection against various viruses (Vandenbussche et al., 2004). In some cases the transfected plants appeared damaged if the expression of the RIP gene was above a certain level, as it was observed with PAP (Lodge et al., 1993; Dai et al., 2003) or with the JIP60 RIP (Görschen et al., 1997).

Plants were transfected with RIP genes to increase their resistance to fungi (reviews by Ng, 2004; Theis and Stahl, 2004). Transgenic tobacco plants transfected with a RIP from barley (Logemann et al., 1992) or with the maize ribosome-inactivating protein b-32 (Maddaloni et al., 1997) acquired an increased resistance to *Rhizoctonia solani* infection, in the latter case without damage to the plants.

Ricin and saporin, were the first RIPs found to be toxic when fed to insect larvae (Gatehouse et al., 1990), and subsequently several investigations were performed to ascertain whether transfection with RIP genes could protect plants from insect infestation. An increased resistance to various insects was observed in plants transfected with genes of RIPs, both type 1 and type 2, e.g. tobacco plants transfected with maize RIP (Dowd et al., 2003, 2006) SNA-I′ or *S. nigra* agglutinin SNA-1′ (Shahidi-Noghabi et al., 2009). Also, resistance to insects was obtained in maize plants by enhancing the expression of an endogenous RIP (Dowd et al., 2012).

RIPs appeared, or their expression was enhanced, in plants treated with jasmonate (Reinbothe et al., 1994) or abscisic acid (Muller et al., 1997), and in plants exposed to unfavorable conditions such as infection by microorganisms (Wong et al., 1995), viruses (Girbés et al., 1996) or fungi (Xu et al., 2007), or subjected to various abiotic stresses: heat and osmotic stress (Stirpe et al., 1996), salinity (Rippmann et al., 1997), mechanical injury (Song et al., 2000; Tartarini et al., 2010).

More recently, new possible applications were suggested when it was found that the expression of barley *Hva1* gene in transgenic mulberry displays enhanced tolerance against drought, salinity and cold stress (Lal et al., 2008; Checker et al., 2011). Also, it was found that the rice genome includes 31 genes for RIPs expressed at various levels in different parts of rice plants, and that the expression of some of these RIPs was enhanced under various stressful conditions, namely infections, cold and salinity (Jiang et al., 2008). The same investigators inserted the gene for one of these RIPs, designated OSRIP18, in an ectopic position of DNA in the rice genome. Interestingly, the resulting overexpression of this gene in young rice plants rendered them resistant to drought and salinity without any apparent damage to the plants (Jiang et al., 2012). The mechanism through which this protection is exerted is unknown. The authors suggested some possibilities, such as a re-organization of protein metabolism through RIP-induced inhibition of protein synthesis, or the effect of one of the other enzymatic activities of RIPs which have been reported, notably the reported superoxide dismutase activity (Li et al., 1997), which could reduce the damage caused by the accumulation of reactive oxygen species occurring in some environmental stresses (Foyer and Noctor, 2005).

Summing up from a practical point of view it seems that enhancing the expression of RIPs in transfected plants might render them more resistant to infections, infestations, and abiotic stress, with considerable economic advantages if the results will be confirmed in the field. An obstacle might be the damage to plants, which in some cases was observed, and which may depend on the plant, the RIP, the promoter used, the extent of RIP expression, all factors that should be investigated.

3. Conclusions

As often occurs in science, new findings leave several questions unanswered, and pose new ones. No precise biological role has yet been assigned to RIPs, although it is common belief that they should be important proteins and give an evolutionary advantage to plants, to justify their conservation. It has been suggested that RIPs could be storage proteins, have a function in the defense of plants against predators, fungi, and viruses (review by Girbés et al., 2004) and be involved in the process of plant senescence (Stirpe et al., 1996). On the basis of their results, Jiang et al. (2012) suggested that RIPs might afford some protection to plants under unfavorable situations. Summing up, it seems likely that RIPs play important roles in the adaptation and defense of plants to pathogens and environmental stress.

One could go forward, recalling that RIPs remove adenine also from DNA and other polynucleotides (Barbieri et al., 1997). A similar activity was detected in *in vitro* cultured mammalian cells, which, like RIPs in stressed plants, appeared higher in cells stressed by lack of serum or by polio virus infection (Barbieri et al., 2001). If these results will be confirmed, one wonders whether the expression of this "RIP-like" activity could be artificially enhanced in mammalian cells and possibly in animals, and what would be the resulting effect.

Finally, research on RIPs was initiated for the sake of knowledge: its developments seem to confirm that *"Fundamental research, sooner or later, leads to important practical applications"* (Krebs, 1981).

Ethical statements

Not applicable, there are no experiments.

Conflict of interest

No.

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