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Fungal immunomodulatory proteins in the context of biomedicine

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1. ABSTRACT

Fungi represent a large group of biodiverse microorganisms with potential applications ranging from industrial fields to the treatment for human diseases. A large number of pharmacologically active compounds including terpenoids, polysaccharides and proteins have been derived from these microorganisms. Fungal Immunomodulatory Proteins (FIPs) are a group of active compounds that are being considered for the treatment of asthma, allergy, autoimmune diseases and cancer. Here, we discuss the discovery, heterologous production bioactive mechanisms of action and their potential use in biomedicine.

2. INTRODUCTION

There is a growing interest worldwide from the academic and scientific community in search of biologically active compounds derived from plants, microorganisms, marine organisms and fungi as macromycetes. They can be used as efficient drug therapies or as aids in the treatment of various diseases as efficient adjuvant agents, decreasing side effects and adverse reactions expressed over other types of drugs (1). This has stimulated the identification and evaluation of a number of natural compounds throughout both *in vivo* and *in vitro* experimental assays of different secondary metabolites and proteins derived from such organisms. Fungus, in particular mushrooms, offer an interesting opportunity for the search of new therapeutic compounds, due to their amount, diversity, culture and manageable genomes, compared with plants.

For ancient times, medicinal fungi have been an abundant source of bio compounds with multiple beneficial effects on human health. Mueller and Schmith (2007) suggest that the traditional estimate of the number of fungal species in about 1.5 million species is still very modest and does not include such species from complex environmental samples, in many cases without fully description (2). From the species that presumably exist, only about 5 to 7 % are formally

described. However, some more recent estimation, suggest that there are in total about 5.1 million species of fungi, surpassing plant biodiversity in proportions that may exceed the ratio of 11:1 in the near future (3).

Among the fungi described, higher fungi comprise four main phyla: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota, whose species have chitinous cell walls, a fundamental difference with plant cells (4).

In particular, the species within the Ascomycota and Basidiomycota divisions considered from the point of evolutionary and phylogenetic areas, the main representatives of the "higher fungi". Among these, the macromycetes offer one of the best possibilities for culinary, as well as therapeutic applications, due to their richness and complexity of bioactive compounds. Recently, there are about 700 recognized species of Basidiomycetes, presumably, with significant pharmacological activities (5). Unfortunately, only 5 to 10% of species of fungi for human use can grow efficiently (6).

A very interesting aspect of biotechnological prospection of fungi, specifically in the field of biomedical sciences, derives from the proven fact that many of the models for drug development from natural products correspond to bioactive substances as secondary metabolites coming from them (7). However, even more important is the fact that most of the discovered secondary metabolites in higher fungi have characteristic chemical structures of drugs (5, 7-12).

Macromycetes have also been an excellent source of protein in world cuisine, mainly due to low fat and no cholesterol. In addition, many of their proteins have shown interesting biological activities, as in the case of *fungal immunomodulatory proteins* (FIPs), ribosome inactivating proteins (RIPs), lectins, laccases and other proteins that have become very popular as medicinal sources, primarily acting as immunomodulatory agents, antitumor, antimicrobials, antifungals, antivirals and antioxidants (13).

For the reasons mentioned above, this review is aimed to show a general perspective on the discovery, molecular structure, recombinant expression in the laboratory and biological significance of fungal immunomodulatory proteins, and their potential use in the pharmacological treatment of several important diseases from the standpoint of public health.

3. DISCOVERY

In 1989, Kino *et al*, reported the first fungal immunomodulatory protein (FIP) called Ling Zhi-8 (LZ-8), isolated from the fruiting body of Ling Zhi medicinal fungi (*Ganoderma lucidum*) (14). From that date, six other FIPs in species of the genus *Ganoderma* have been reported: FIP-gts of G. *tsugae* (15), FIP-*gsi* of G. *sinensis (*16), FIP -gja of G. *japonicum* (Genbank: AAX9824), FIP-gmi G. *Microsporum* (17), FIP-gas of G. *atrum* (GenBank: KM077027.1) and FIP-gap G. *applanatum* (Genbank: AEP68179). Other eleven fungal immunomodulatory proteins have been described at molecular or sequence level in other Basidiomycetes: FIP-fve *of Flammulina velutipes* (18), FIP-vvo of straw mushroom (19), FIP-Apl of *Auricularia polytricha* (20), FIP-aca of *Antrodia camphorate* (21), FIP-PCIP *Pleurotus citrinopileatus* (22), FIP-PCP of *Poria cocos* (23), FIP-tvc of *Trametes versicolor* (24,25), FIP-ppl Postia placenta (26), FIP-cru Chroogomphis rutilus (27), FIP-Lrh of *Lignosus rhinocerotis (*Tiger milk mushroom) (28), FIP-tfu of *Tremella fuciformis* (GenBank: EF152774) and FIP-nha *Nectria haemotococca* edge Ascomycetes (29).

Subsequently, roughly around other sixteen putative FIPs have been reported from different edible or medicinal mushrooms at the nucleic acids sequence level. For example, recent genome sequencing of *Lignosus rhinocerotis* revealed two putative FIPs (GME7566_g and GME10641_g), named FIP-Lrh, both exhibited 64% identity to FIP-glu (30).

In spite of the study of twenty FIPs since the first FIP was isolated from *Ganoderma lucidum*, today, only five proteins at the crystallographic level are reported as accessions in the Protein Data Bank (PDB).

4. GENE STRUCTURE AND PROTEIN CHARACTERISTICS

FIPs identified and isolated from edible and medicinal macromycetes, constitute a family of proteins characterized by high structural and functional similarity. In addition, this family shares certain level of sequence similarity with the immunoglobulin protein family. Phylogenetic analysis can be used to test if both protein families may have originated from the same common ancestor, as it has been hypothesized since its discovery of LZ-8 (31).

It is noticeable that among the different FIPs, their primary structure had 60–70% similarity, and the sequences of LZ-8 (FIP-glu) and FIP-gts even shared the same amino acids (15). Interestingly, most of them exhibit homology to the family of the immunoglobulins (Ig) and phytohaemagglutinin, as they have high similarity of sequence in the variable region in the heavy chain of immunoglobulins (28, 31-33).

Sequence analysis, reveals that FIPs have similar structure and immunoregulatory activity to phytohaemagglutinin and immunoglobulins. Taking into

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consideration that the fungal and the animal kingdom are related as they both belong to the Ophistokonts, with a common ancestor existing about 1 billion years ago (34-35); the idea that the FIPs family and the mammal immunoglobulins family may conserve a substantial proportion of similarities and related functions, results attractive. This is applicable to some physiological mechanisms; for example, in response to various abiotic or biotic signals as filamentous fungi produce small signaling and/or defensive bioactive molecules (28, 36-37). This mechanism is equivalent to some molecular responses of immune systems in vertebrates.

Another interesting evolutionary relationship between fungi and animals emerge from the analysis of the Velvet protein family. These proteins are involved in the regulation of the secondary metabolism as well as the control of tissue growth and differentiation in most clades of the fungal kingdom from chytrids to basidiomycetes, including the model mold *Aspergillus nidulans* (37-38). The structure of the Velvet domain is strongly reminiscent of the N-terminal immunoglobulin-like domain found in the mammalian transcription factor NFkBp50. It is proposed that, like NFkB, various homo- or heterodimers of Velvet proteins, modulate gene expression to drive development and defensive pathways in fungi. Taken together, this suggests a common functional origin for the coordination of fungal development with secondary metabolism and the immuno-inflammatory response control in vertebrates, including humans (39).

It will be interesting to determine what additional common features exist between fungal and animal proteins and genes. Recently, it has been shown that the physical interactions between the two deneddylases: the COP9 signalosome and DEN1/DenA, a developmental regulator protein, are conserved between humans and fungi; COP9 signalosome supports proteasome-dependent protein degradation of DEN1/DenA in fungi as well as in human cells (39-40).

Ganoderma, FIP LZ-8 was the first fungal immunomodulatory protein that was isolated and characterized, extracted from *Ganoderma lucidum* (Genbank Accession No. M58032.1) (31). Complete gene sequence encoding for FIP LZ-8 consists of 1029 bp. This gene has a promoter region with TATA motif (TATAAA) located 88 nucleotides upstream the consensus site at transcription initiation. The position of the promoter is similar to that reported when the gene is present in filamentous fungi and yeasts, in which there are two repeated sequences (GCAATTC), 80 nucleotides upstream of the promoter sequence, which acts as a proximal determinant element involved in transcription intensity control. The 5'-UTR no coding region has a length of 119 bp, single intron is located within this sequence with a length of 61 bp; the open reading frame is located between 527 to 866 nucleotides, including the start codon ATG and the stop codon TAG. The polyadenylation signal, useful in determining mRNA stability in the cytoplasm, is at nucleotide position 996 (28, 31). Due to their small size, FIPs can be easily modified at the molecular level, with potential use in a wide-range of industrial applications.

In the fungus *Ganoderma, sinense* the complete sequence of the gene FIP-gsi has a length of 1072 bp (16). Both, the gene sequence and the location of the structural and regulatory elements, are similar to those reported for FIP LZ-8 in *G. lucidum*.

Description of this gene at the sequence and structural level is useful for comparative analysis with respect to other sequences. In a recent work, 11 different immunomodulatory proteins downloaded from the NCBI website were used to perform a sequence alignment between them and the two proteins GL18769 and GL18770, annotated in NCBI as gi|126657 and gi|187961980, respectively. These proteins were identified as likely immunomodulatory proteins from a proteomic analysis in *Ganoderma lucidum* (41). The two proteins showed a high similarity with the 11 proteins reported in GeneBank. From this analysis, it could be suggested that these proteins shared the same conserved amino acid sequence responsible for the bioactivity, which certainly could aid in immunomodulatory protein engineering in the future.

The different cDNA sequences and genomic DNA of several FIPs reported in Genbank have an open reading frame between 330 to 350 bp, which determines that this protein family consists of 110 to 116 amino acids and a molecular weight of 12.4 to 15 KDa (33). When aligning amino acid sequences of different species, several FIPs showed a high level of homology in their primary structure (28). This suggests that it is a highly conserved protein on its molecular evolution and that may be necessary for the growth and development of the fungus (25). These proteins are rich in the aminoacids aspartate, histidine and valine, but characterized by lack of cysteine and methionine (33).

There is a report that FIP LZ-8 is a glycoprotein containing 1.3% carbohydrate, although, in other FIP isolated by chemical methods in other macromycetes genus not glycosylation reported in their structure; however, there was glycosylation observed in some FIPs expressed by means of recombinant technologies in eukaryotic systems (42-43).

Secondary structure analysis performed in FIP-glu LZ-8 (from *Ganoderma lucidum*), FIP-gsi (from

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Ganoderma sinensis), FIP-gmi (from *Ganoderma microsporum*), and FIP-fve (from *Flammulina velutipes*), indicates that the N-terminus has one α-helix constituted by 13 amino acids that stabilize the protein by hydrophobic interactions (43). This favors its homodimeric structure and its immunomodulatory activity, such as activation of interleukin 2 (IL-2) and interferon (INF-γ) (43-45). The FIPs also exhibit seven β sheets with conserved amino acids sequences, which are essential for the recognition of cell surface (23, 33, and 46).

The importance of hydrophobic interactions for the formation of homodimer in FIP-gts (from *Ganoderma tsugae*) was demonstrated by Lin *et al*. (1997) when conducting an experiment in two-hybrid yeast system; for which they designed various constructions exhibiting deletions in the amino acids constituting the α-helix N-terminus. The mutant proteins showed absence of 13 amino acids and hydrophobic amino acids located at positions 5, 7 and 9, and that corresponds to leucine, phenylalanine and leucine, respectively (15). When testing double hybrid these were unable to join the wild type phenotype of FIP-gts to form the dimer, which led to the conclusion that the quaternary structure of the FIP-gts may be the way to trigger the immunomodulatory response in the cells of vertebrates throughout mechanisms of signal transduction (15).

5. BIOACTIVITY IN MODEL SYSTEMS

Many fungi, especially the mushrooms, have been intensely studied as important sources of natural compounds, including secondary metabolites and peptides like FIPs, with many potential effects in human health, as well as other applications including promising bioactivities for commercial developments (11-12, 47-58)

Since the discovery of FIPs, several immunomodulatory effects with promising beneficial potentialities in human health have been reported (59). For this reason, several FIPs, including LZ-8, are considered good candidates for the development of functional food supplements or therapeutic agents for use in the prevention or treatment of cancer and autoimmune diseases (43, 60-61).

Despite of being highly conserved in primary sequence and structure, FIPs family members have disparate biological activities (62). For example, aggregation of FIPs is commonly observed in red blood cells, and different FIPs uniquely hemagglutinate various red blood cells (14, 18). Using *in situ* experiments, it has been broadly demonstrated that different FIPs are able to stimulate mouse splenocytes proliferation (63) and human peripheral blood lymphocytes (hPBLs) (31,59). These effects are more commonly related with changes in the expression of several cytokines, including IL-2, IFN-γ, and TNF-α (44).

In a number of experiments *in vivo,* several FIPs exhibit preventive effects on systemic anaphylactic reactions and significantly decrease mouse footpad edema during the Arthus reaction (64). In addition to immunomodulatory activities, antitumor functions of FIPs have been researched extensively (65).

Many studies are focused mainly on the elucidation of immunity factors like cytokines, as a response to either crude extracts or specific compounds or toxins produced naturally by fungi. For example, *Ganoderma lucidum* fraction, with high content of polysaccharides activate the expression of a series of cytokines like IL-1β, IL-2, IL-12, TNF-α, and IFN-γ, but reduces IL-4 and IL-10 release in mice cells (66).

According to these evidences, that is to say, the immunoglobulin superfamily could be traced back to fungi. In this regard, it is almost obvious and very interesting to study whether there is any evolutionary relationship between the activity mechanisms of immunoglobulins in vertebrates and the immunomodulatory activity exerted by FIPs in animal cells. In fact, recently Ipcho *et al*. (2016) found strong evidence that plants and animals detect bacterial presence through Microbe-Associated Molecular Patterns (MAMPs); which induce an innate immune response in these taxa. However, little is known at the molecular level about the interactions between fungi and their beneficial or pathogenic invaders, and less is known in relation to the mechanisms that explain how fungi detect and differentiate its microbial allies from their natural enemies (67).

Nonetheless, experimentally it has been demonstrated that most FIPs have the ability to enhance the immune response in a number of vertebrate's model systems. This is presumably by means of stimulating the expression of important cytokines and molecular factors related with the immune response, including IL-2, IL-4, IL-12, TNF-α, and IFN-γ, among others (33, 43, 52, 68-69). The evidence for the mechanisms was gathered using several model organisms, including humans and other vertebrates (68). The FIPs can be also useful products for the treatment or prevention of autoimmune diseases and cancer, among others. Also to relieve pain or side effects caused by drugs in cancer patients (60- 61, 70). For these reasons, the FIPs should be consider good candidates for new developments in the field of functional foods as well as in the pharmaceutical industry.

The FIPs have been isolated from both, the haploid mycelium, and from the diploid fruiting body of various macromycetes fungi, particularly from the

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phylum Basidiomycota. For several FIPs there is an experimental description of the basic DNA encoding sequence of the gene, and the primary, secondary and tertiary conformation of the protein (33, 43, 71). There are also insights about their effects on the immune system of vertebrate (29, 69), however it is not known in depth the molecular mechanism that triggers the immunomodulatory response (26, 72).

For example, some FIPs have an important effect at the cellular level of defense mechanisms at the immune system in model vertebrates. For instance, it has been demonstrated that FIP-*pcp* of *Poria cocos* is a key factor in signaling through TLR4 in peritoneal macrophages and induce co-stimulatory signals of CD3/CD28, increasing significantly the surface expression of CD44 and CD69 on effector T cells. FIP-*pcp* could also upregulate T-bet and STAT4 expressions and IFN-γ and IL-2 secretions. Oral administration of FIP-*pcp* suppressed the production of both total and OVA-specific IgG1 in serum and enhanced the amounts of serum and OVA-specific IgG2a and Th1-related cytokine production in BALB/c splenocytes. In addition, oral administration of FIP-PCP significantly reduced IL-4 and IgE expressions in a murine model of atopic dermatitis. These results provide evidence that FIP-*pcp* could regulate mammalian immune cells and reveal their pharmaceutical potential in developing therapeutic strategies against Th2-mediated immune disorders (73).

6. EXPRESSION OF RECOMBINANT FIPS

One of the prominent limitations when it comes to take advantage of the health benefits attributed to FIPs extracted directly from the fungus tissue is that the concentration of these proteins is very low when direct chemical extraction methods are used; moreover, a large amount of biomass for its extraction is required. Recently, fermentation of *Ganoderma lucidium* mycelia and purification of LZ-8 represents the main method in order to obtain LZ-8, but this process is costly, time consuming, and results in a low yield (60). For example, direct extraction of native FIPs from mushrooms resulted costly and time-consuming; approximately 5 to 10 mg of purified FIP LZ-8 was obtained from 340 g of mycelia (14). This problem becomes an important issue when attempting to get an industrial production of the fungal proteins (72,74).

In order to overcome this back draw, it has been suggested since the late 90s, the use of recombinant protein technologies (75). For this purpose, different expression systems have been evaluated, both in prokaryotic and in eukaryotic cells; all of them overcome some of the difficulties related to the direct chemical extraction methods, such as contamination with other undesirable fungi cellular proteins and limiting native concentrations impede scaling to industrial level in order to increase production (60). Quantitatively, in the biopharmaceuticals industry, microbial production of recombinant proteins still predominates over mammalian-based expression systems. For example, the total biopharmaceutical manufacturing activity in 2010 equated to some 26.4 metric tons (26,400 kg) of pure protein (active pharmaceutical ingredients) of which some 17.9 metric tons (68 %) were derived from microbial systems, with the remaining 8.5 metric tons (32 %) derived from mammalian systems (56).

In the next sections, we will describe some selected and illustrative examples of FIPs production under the most common recombinant protein methodologies.

6.1. FIPs production in bacteria

Escherichia coli is the most commonly gram negative bacteria used as an expression system of recombinant proteins for therapeutic use in humans, such as insulin, growth hormone and parathyroid hormone, among others (76-77). *E. coli* is very popular for recombinant DNA technology due to the extensive knowledge of its genomic organization, genetic regulation machinery, metabolism and physiology, which facilitates the genetic manipulations required (78). Also, transformation with exogenous DNA in this bacterium is relatively simple and quick, due in part to their accelerated growth rate in standard growing media; high productivity of the recombinant proteins and a low production cost (78). Therefore, there is a wide variety of *E. coli* strains and plasmids available on the market designed to promote the expression and purification of recombinant proteins (78). Despite its undeniable advantages, this expression system may present contamination by lipopolysaccharide (LPS) that trigger immune response in humans and other mammals (76,79). It lacks cellular components for post-translational modifications (required by a protein of eukaryotic origin) and for secretion of the protein, often generating inclusion bodies that require special technical protocols for the purification and recovery of the protein of interest (76,79).

In *E. coli,* several fungal immunomodulatory proteins, including FIP-LZ8, FIP-fve, FIP-nha, FIP-tvc and FIP-gsi, have been expressed, with different yield percentages of soluble protein (79-80). Differences in gene regulation mechanisms in different species of macromycetes, as well as unequal efficiencies in recombinant vectors or *E. coli* strains or both, may explain in part this effect (79-80).

The immunomodulatory activity of FIPs expressed in *E. coli*, implemented with commercial recombinant systems, have demonstrated being adequate in most of the cases, except for the reFIP-fve

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(25,75). Surprisingly in this case, it showed a higher activity when extracted directly from the fruiting body of the fungus *Flammulina velutipes*, than that expressed by means of the recombinant *E. coli* system. According to these authors, this effect could probably be due to inappropriate protein folding under the expression system used in this experiment (75)

Besides *E. coli*, there have been used *Bacillus subtilis* and *Lactoccocus lactis* for heterologous expression of FIP LZ-8 (60). Nowadays, the genetic engineering technologies available, allow the easy manipulation of these expression systems as well; they provide an additional advantage over *E. coli* associated with the faculty to secrete the heterologous proteins, which is useful for the purification stage (60, 81-82). In addition, in the United States, the FDA agency (Food and Drug Administration), recognizes that some of these bacteria strains are safe organisms, regarding the absence of toxic amounts of the dangerous toxins (lipopolysaccharides (LPS)); for this reasons they are considered important tools for the production of therapeutic molecules in biomedicine (81-82).

For instance, the expression analysis *B. subtilis* and *L. lactis* of the reFIP-LZ-8 was used as vectors (60). They compared several expression systems that take advantage of the secretory faculty of the strains used. They evaluated the expression levels under different vector-strain combinations, as well as the biological activity of the recombinant proteins produced, in terms of the capacity for the stimulation of cytokines in different mammal cell lines. These authors showed that reFIP LZ-8 expressed in *B. subtilis* and L. *lactis* is homodimeric, as has been reported previously for this protein, but in this experiment with a slightly different conformation responsible for the increased immunomodulatory capacity. The authors concluded that recombinant LZ-8 exhibited potent antitumor activities and different capacities for modulating Th1 and Th2 cytokines. The recombinant LZ-8 may be useful in immunoprophylaxis for food allergies and other allergic diseases or cancer therapy. The recombinant LZ-8 purified from *B. subtilis* can be used as a food supplement, while optimization of expression and secretion efficiency in food-grade *L. lactis* will provide a useful source of recombinant LZ-8 for safe use as an orally administered agent (60)

6.2. FIPs production in yeast

Nowadays, the yeast *Pichia pastoris* is one of the most popular systems for the production of recombinant proteins; as a methylotrophic, this yeast uses methanol as the inductor of expression (83). *P. pastoris* possesses several advantages over other expression systems. It presents a rapid growth media culture, absence of contamination by endotoxins and bacteriophages; furthermore, the fermentation and culture medium system for the growing of the yeast result simple and economic and is suitable for large-scale industrial production. Due to the gene structure of the vectors employed *P. pastoris*, it has the ability to post-translationally modify, fold and secrete heterologous proteins into the media; allowing an easier purification process (83-84).

There are several examples with *P. pastoris* used as a system for heterologous expression of reFIPs; the results show a good performance and stable percentage of biological activity (71). Lin *et al*. (2013) cloned and expressed reFIP-fve in *P. pastoris;* reaching a yield between 191.2 to 258.2 mg/l, as assessed by quantitative analysis (85). This is significantly higher than that reported for reFIP-fve and reFIP-gts expressed in *E. coli* and insect cells with a yield of 5 mg/l and 20 mg/l, respectively (15,75). Additionally, recent efforts promise that reFIPs are being scaled-up to industrial production in a 100-l fermentation tan with the yield over 800 mg/l (46, 85).

6.3. FIPs production in eukaryotic cells

In an effort to improve the quality and the bioactivity of recombinant proteins, in a series of cases it has been used successfully Sf21from insect cell lines. These cells derive from the insect *Spodoptera frugiperda,* from where cells were isolated for the first time in 1977. The cell line is manipulated to obtain recombinant proteins using baculovirus as the expression vector, which does not infect vertebrates, and therefore does not generate toxic or pathogenic compounds that may affect the human health (86). Among the principal advantages of Sf21 cells include simplicity of manipulation, eukaryotic-like post-translational maturation, and ability to produce quaternary structure in the recombinant proteins generated. Recent experiments pointed out that their main disadvantages are associated with slow growth, which increases costs; and risk of contamination of the cell cultures by rhabdovirus in commercial lines, affecting biosecurity for industrial applications (13,43,86).

In 2006, Jinn *et al*. cloned the gene FIP-gts from *Ganoderma tsugae* on a PAcP10 vector to generate a recombinant baculovirus, which co-transfected Sf21 insect cells with the aim of obtaining the heterologous expression of rFIP-gts and its possible use for oral administration. The experimental design involved the *E. coli* protein expression system in order to compare the efficiency of protein produced in both systems. Optimal expression of rFIP-gts in Sf21 cells was observed, and the yield was up to 47.2 μg/3x10^6 infected cells. The immunomodulatory activity of the purified rFIP-gts was detected as the induction of interleukin 2 released from murine splenocytes. Compared with the rFIP-gts produced in *E. coli* cells,

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the rFIP-gts produced in Sf21 cells possessed evidently higher specific immunomodulatory activity. This suggests that the lack of posttranslational modification in bacteria may affect the folding and bioactivity of rFIPs produced in prokaryotic systems. Regarding the possibility of oral administration of rFIP-gts as possible therapeutic agents during pharmaceutical treatment of diseases like cancer and autoimmune diseases, the Sf21-Baculovirus recombinant protein system at scalable production appears as a very attractive alternative (87). However, other publications suggest that rFIP-gts showed a short half-life, probably due to proteolytic degradation process (13,43).

A comparative analysis of the synthesis of rFIP-gts transfected with baculovirus between Sf21 cells and insect larvae *Trichoplusia ni* (inoculated by aerosol), showed that both models correctly express the FIP-gts with a very similar efficiency. It is suggested that the use of *Trichoplusia ni* larvae, inoculated with aerosol, can reduce the time and costs of rFIP-gts production for biomedical applications (88).

A rFIP-fve produced also in Sf21 cells exhibited high expression levels (6.25 mg/l, equivalent to 3.1 μg/1x10^6 cells) when transfected with baculovirus carrying the expression vector vAcP10SPbbxFve; however, it was found that 96 hours after infection there was breakdown of insect cells and recombinant protein degradation (89). Nevertheless, this is higher performance than that obtained when using *E. coli* as expression system (5.0 mg/l) (75).

6.4 FIPs production in basidiomycetes

Since the identification of FIPs, some efforts have been made using basidiomycetes as an alternative model for the production of these recombinant peptides; also, other compounds of pharmacological interest present in the macromycetes whose production could be scaled at industrial level and at lower cost under certain conditions (70). For instance, *Coprinopsis cinerea* is a small macromycete, whose life cycle is completed in two weeks. It can be easily grown in the laboratory in defined media and engineered by mutation or transformation at all stages of development, making it an experimental model for the study of multicellular development (90-91). For example, using this heterologous peptide production system, FIP-gsi was expressed in *C. cinerea*, co-transformed with plasmids pCc1001 and pBfip.gsi, purified from fresh mycelium, and lyophilized. The recombinant protein was produced with a yield of 314 mg/kg (70).

However, working with basidiomycetes and filamentous fungi in cell culture may bring some problems; for example, oxygenation of cultures employing fragile cells, such as animal cells and filamentous fungi, is often problematic, as sparging and agitation are limited to shear stresses that are not harmful. Typical energy dissipation rates in bioreactors are usually below those deleterious to animal cells; thus, damage from agitation should not be expected (74).

6.5. FIPs production in plant cells:

For the production of recombinant proteins with applications in biomedicine, it has been found useful the use of plant cells as an efficient expression system compared with others (92- 95). It offers several advantages over other systems, including the low cost of biomass production, high scalability and good biosecurity due, in theory, to the lack of pathogens that may be transmitted to humans or animals affecting their health (92).

However, the post-translational modification made in the Golgi apparatus of plant cells, differs from animals because plants do not have the capacity to produce some carbohydrates present in human glycoproteins such as β-1, 4 galactose and sialic acid. Instead, plant cells add monosaccharides such as xylose and α-1,3 fucose, which may alter the bioactivity of the molecule and trigger immunogenicity in humans (92).

Su *et al*, 2012 transformed rice suspension cells by means of *Agrobacterium tumefaciens* to express the fusion protein Der p 2-FVE; which consisted of FIP-fve fused with a major allergen from the mite body *Dermatophagoides pteronyssinus* (Der p 2). Rice transformed cells produced 7.5 mg/l of protein, corresponding to 10.5 percent of the secreted proteins. The evaluation of the biological activity *in vivo* and *in vitro* suggested that this as a viable system for protein production with immunotherapeutic potential for treatment of allergic diseases, as it potentiates the immune response (96).

Cong *et al*. (2014) cloned the promoter of the gene coding for FIP-fve using the methodology of chromosome walking in order to understand expression regulation mechanisms of FIP-fve. The expression vector contained the GUS reporter gene (β-glucuronidase) and GFP (Green Fluorescent Protein). They performed a transformation on tobacco leaf discs with *Agrobacterium tumefaciens.* Evaluation of the transformed plants showed that the promoter of FIP-fve induced expression of the reporter genes, opening the possibility that transgenic plants can be used for the production of FIPs (97).

6.6. FIPs production by means of transgenic microalgal technology:

Bacterial and yeast-based bioreactors are the most widely used systems for the production

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of recombinant proteins since they are very well characterized, because their genomes are easy to manipulate, and their cultivation is simple and inexpensive (98- 100). Bacteria, however, do not perform the post-transcriptional and post-translational modifications, including glycosylation, phosphorylation and disulfide bond formation, required for the correct folding and assembly of more complex proteins. This is important in relation with the need for production of human therapeutic peptides by means of biotechnology using heterologous protein systems in bioreactors. In this case, other models including yeasts may represent an attractive alternative approach, because eukaryotic yeasts can perform these modifications; but again, there is a limitation; most of the times their profiles are unsuitable for therapeutic proteins for animal or human consumption. The main reason for this is that recombinant proteins in yeast are usually hyperglycosylated, which alters immunogenic epitopes, and the high-mannose glycosylation performed in such systems results in low *in vivo* half-life of proteins: Both factors in many instances may compromise the therapeutic activity of the products (99-100).

On the other hand, plant-based reactors, even though are much less expensive than their mammalian and insect counterparts and resistant to most animal infecting pathogens, have slow growth cycles and are linked with concerns relating to environmental contamination by genetically modified plants. Although differences are observed in glycosylation profiles between animal and plant cells, in many cases the stability, correct folding and resistance to proteases of mammalian-derived products have not been significantly affected. However, the major drawback of therapeutic applications of any plant derived from recombinant protein is plant-specific protein glycosylation, which may cause allergic reactions in patients. Plant-derived proteoglycans have been linked to allergic reactions, which is a major concern for recombinant proteins destined for human therapeutic applications (101,103)

Although transgenic microalgal technology is still in its infancy, microalgae may represent the best of both worlds by combining simple inexpensive growth requirements, with a rapid growth rate and potential for high-density culture of microorganisms, and capabilities for post-transcriptional and post-translational processing of plants, without any known toxic side effect (104). However, the development of economically viable microalgal expression systems is hindered by low recombinant protein yields (99,104).

As pointed out, the production of recombinant pharmaceutical proteins in heterologous systems has increased significantly and there are a number of commercial alternatives available. Most applications in human health involve complex proteins and glycoproteins that are difficult to produce, thus promoting the development and improvement of a wide range of production platforms. This is having an important impact in the market for recombinant pharmaceutical proteins, which explains its rapid expansion in the last few years (2, 56,105 - 107). Yet, no individual system is optimal for the production of all recombinant proteins: So, the diversity of platforms should be considered and evaluated carefully, before a major endeavor with prospects for scalability is going to be attempted.

7. POTENTIAL IN BIOMEDICINE

Regardless of the methodology used for the production and purification of fungal immunomodulatory proteins from different species of macromycetes, all research on FIPs include evaluations of the biological activity in different model cell lines and laboratory organisms. They fund bioactivities mainly related with clumping of red blood cells, induction of cytokine expression, antitumor properties, anti-anaphylaxis, and anti-inflammatory activity, among others. This provides an advance in the knowledge of the immunomodulatory effects and potential applications of this protein family as a promise for their use as adjuvants for the treatment of cancer, inflammatory diseases and immunodeficiency, among others. In the next part, we will mention some research reports dealing with important bioactivities of the FIPs in relation with human health supported by experimental evidence.

7.1. Hemagglutination:

Hemagglutination assays promoted in vertebrates by FIPs usually make use of tests in whole blood samples taken from sheep, rabbit, rat, mouse or human. *In vitro* assays of biological activity indicated that the rFIP-nha obtained from ascomycete *Nectria haematococca* caused hemagglutination of human and rabbit red blood cells, significantly stimulated mouse spleen lymphocyte proliferation, and enhanced expression of interleukin-2 (IL-2) released from mouse splenocytes, revealing a strong antitumor effect against several human cell lines, including HL60, HepG2 and MGC823 (29).

Recently, a novel FIP gene from *Chroogomphis rutilus* (FIP-cru) was cloned, analyzed, and effectively expressed in *P. pastoris* GS115 (27). The recombinant rFIP-cru exhibited bioactivities, including the agglutination of mouse and sheep blood cells, the induction of murine splenocyte proliferation, and the enhancement of IL-2 secretion. With regard to its bioactivity, interestingly rFIP-cru can agglutinate mouse and sheep red blood cells but not human red blood cells. It is suggested that rFIP-cru can be effectively produced in its bioactive form with the

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P. pastoris expression system (27). Similar to other FIPs, rFIP-cru preliminarily exhibits certain biological activities *in vitro*. Given its basic properties, rFIP-cru probably possesses other immune-stimulating properties, which require further investigation (27).

7.2. Cytokine expression

Cytokines are proteins produced by various cells of the immune system that typically exhibit pleiotropic effects on a number of mechanisms mainly related with regulation of the immune response, development of the hematopoietic cell lineage and cell-cell communication, among others. They are highly localized in soluble signaling proteins produced by many cells of the immune system (neutrophils, monocytes, macrophages, B-cells, and T-cells) to regulate immune responses. There are several different families of cytokines and the number continue to grow. For example, the interleukin family is categorized in numerical order (up to IL-38) and other families include those describing functional activity such as the tumor necrosis family. Among the most studied cytokines are the interleukins (IL), tumor necrosis factor (TNF), interferons (INF), colony stimulating factors (CSF) and chemokines. Cytokines differ, in their not only function, but also have a wide variety of molecular weight ranges from approximately 6 to 70 kDa (108).

Studies of cytokine expression induced in model cells and organisms by means of FIP administration are mainly based on techniques including RT-PCR and ELISA tests. They are frequently performed in mouse spleen cells. In order to test FIPs bioactivity the protocols generally use cell lines grown in appropriate media, for example RPMI 1640 supplemented with fetal bovine serum and antibiotics. Suspended cells are then incubated with different concentrations of FIPs in order to assess production levels of different cytokines. In cases in which RT-PCR is applied, the expression of different interleukins as IL-2, IL-4, IL-6, interferon gamma (INF-γ) and tumor necrosis factor (TNF) are analyzed in a quantitative manner. In the case of expression analyzes by ELISA, the techniques are limited by the availability of appropriated antibodies and the results are mainly qualitative (25-26, 44, 46, 80, 85) .

Consistently, it has been observed that FIPs of different species of fungi promote the expression of different cytokine profiles, either in relation with the dosage used or with the molecular modification and structural conformation adopted. For instance, Fip-vvo purified from crude extracts of *Volvariella volvacea* cell cultures markedly enhanced the expression of interleukins IL-2, IL-4, TNF-a, IFN-c, LT and IL-2R, but not IL-1, IL-3, IL-5 or IL-6, tested in a dose-dependent fashion using mouse spleen cells and human peripheral blood lymphocytes (19). The proliferative response induced by Fip-vvo was similar to that induced by LZ-8 and Fip-fve; however, Fip-vvo is 20-fold more potent than Fip-fve on lymphocyte activation (18-19, 75).

In other study, recombinant protein rFIP-gts cloned from *Ganoderma sinensis* and produced in *Escherichia coli* expression system, was able to induce significant amounts of IL-2, IL-3, IL-4, IFN-γ, TNF-α and IL-2R, but not in the case of IL-1α, IL-5, IL-6, and LT (80). This suggests that rFIP-gts is mainly acting on Th1 cells and less on Th2 cells, which is in agreement with the effects of FIP-vvo, which could enhance transcriptional expression of IL-2, IL-4, IFN-γ, TNF-α, LT, and IL-2R (19).

When rFip-gts was produced in the insect cell model *Trichoplusia ni* inoculated with recombinant baculovirus, it possesses the same power as LZ-8 in inducing IL-2 in murine splenocytes (88). In contrast, in the same study the level of IL-2 induction resulting from the non-glycosylated rFip-gts was lower than the glycosylated form of the recombinant protein. Evidently, the glycosylated form of rFip-gts produced in *Trichoplusia ni* larva possesses a higher specific immunomodulatory activity than those non-glycosylated rFip-gts. The results also indicated that the post-translation processing of rFip-gts could play an important role in enhancing and maintaining the required immunomodulatory activity exerted by rFip-gts on the T lymphocytes (88).

These experiments illustrate that the differences presented by the various FIPs with respect to differences in expression levels of cytokines and bioactivities most probably may be related to changes present in the secondary and tertiary structure of these proteins and modifications derived from the host cell used for the expression process.

7.3. Antitumor activity

According to the data gathered from World Health Organization (WHO), cancer has become one of the leading causes of death worldwide (109). This comes to show the necessity of new alternatives that contribute to the curative treatments or acting as efficient adjuvants in order to reduce the incidence of these diseases. Efficient adjuvants should enhance robust and long-lasting adaptive antigen-specific immune responses that confer strong prophylactic and therapeutic antitumor effects.

In this regard, there have been several studies focused on analyzing the potential effects of FIPs in cancer cells, both *in vivo* and *in vitro*, in different cancer cell lines, which would be a starting point for future development of drugs with high efficiency, high specificity and low toxicity (43).

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A prerequisite for an adjuvant to function properly is the activation of innate immunity. The protein FIP-fve obtained from crude extracts of *Flammulina velutipes* cell culture was evaluated by its potential application as an adjuvant for antitumor immunotherapy (110). When the human papillomavirus (HPV) the)- and with 16 E7 oncoprotein, were used in mice coimmunized with HPV-16 E7 and FIP-fve., showed enhanced production of HPV-16 E7- in specific antibodies as well as expansion of HPV-16 E7-specific interferon (IFN)-γ producing $CD4^+$ and $CD8^+$ T cells as compared with mice immunized with HPV-16 E7 alone. In addition, they found that HPV-16 E7 plus FIP-fve co-immunization significantly up-regulated HPV-16 E7-specific IgG1 and IgG2c. Also in this study, FIP-fve induced the splenic dendritic cells phenotypic maturation *in vivo*. Tumor therapeutic assays showed that this treatment significantly prolonged the survival of tumor-bearing mice. Interestingly, FIP-fve could stimulate the maturation of splenic dendritic cells *in vivo* and induce antigen-specific $CD8^+$ T-cell immune responses; making of FIP-fve a potent adjuvant that acts as a protector with properties that enhance T helper type one antigen-specific humoral and cellular immune responses, which confer strong antitumor effects. This opens the possibility of using FIP-fve as an efficient adjuvant, an attractive alternative to the current vaccination strategy for cancer immunotherapy (110).

Recently, a new approach in immunotherapy has been gaining attention; DNA vaccination is a powerful strategy for antigen-specific immunotherapy against various diseases, including cancer; nevertheless, the low efficacy of DNA vaccines in large animals and humans has impaired their practical use (111-112). Therefore, development of novel strategies to increase the DNA vaccine potency is a primary goal in cancer therapy. In this direction, the identification of strong adjuvants appear as a promising opportunity for enhancing the immunogenicity of DNA vaccines.

In this respect, recombinant FIP LZ-8 cloned from *Ganoderma lucidum* and produced in a yeast expression system stimulate mouse bone marrow-derived dendritic cells via TLR4; its stimulatory effect was not due to any microbe contaminant. In addition, FIP LZ-8 enhanced the ability of dendritic cells to induce antigen-specific T cell activation *in vitro* and in a vaccine model *in vivo* (113). Interestingly, in this report FIP LZ-8 co-treatment strongly improved the therapeutic effect of DNA vaccine against MBT-2 tumor cells in mice. The research provides strong evidence that FIP LZ-8-stimulate dendritic cells and can induce antigen-specific T cell activation, both *in vitro* and *in vivo*. The secretion of IL-12 from dendritic cells and the production of IFN-γ from activated T cells indicate that dendritic cells FIP LZ-8-stimulated promote preferentially the pathway on Th1 differentiation (113).

FIPs may also contribute in the fight against cancer in other molecular mechanisms. For example, it is well known that p53 is the most frequently mutated cell cycle suppressor gene in human cancers; this results in the loss of p53's function as a transcriptional factor and as a cellular gatekeeper for growth and division (114). In cancers without p53 mutations, the p53 pathway is often inactivated, which partly occurs via E3 ubiquitin-protein ligase, MDM2 (Murine Double Minute 2), a negative regulator of p53 (115). Activation of the p53 protein protects the organism against the propagation of cells that carry damaged DNA with potentially oncogenic mutations. Because the p53-MDM2 interaction is structurally and biologically well understood, the design of small molecules that disrupt or prevent it, has become an important target for cancer therapy and is currently a hotly pursued therapeutic strategy (116-117).

In order to assess the efficacy of a recombinant FIP from *Ganoderma lucidum* (rLZ-8) as a modulator of this pathway, human lung cancer cells A549, and Lewis lung carcinoma (LLC)-1 xenograft-mice, were used as *in vitro* and *in vivo* models, respectively. It showed that at concentration of 10-15 mcg/ml of cell culture rLZ-8 is a successful anticancer agent that interact with p53 regulating via stabilization in cancer cells via the RPS7-MDM2 pathway, suggesting that rLZ-8 is a potential novel chemoprevention and treatment agent for lung cancer (118). This inhibition was associated with an increased expression of p53, p53, p21/Cip1 and p27/Kip1, generating inhibition and cell cycle arrest G1 stage. In experiments using LLC1 (Lewis Lung Cell), mouse cells were treated with LZ-8 resulting in inhibition of cell proliferation at a similar concentration of that apply to the A549 cell line, proving that p53 is also involved in the inhibitory effect of LLC1 cell growth. Moreover, it was shown that rLZ-8 reduces pre-rRNA expression, which suggests that rLZ-8 may affect pathways associated with either RNA polymerase I activity or the various transcriptional factors that are involved in rRNA transcription in cancer cells (118). All this results have shown that rLZ-8 inhibits the proliferation of lung cancer cells *in vitro* and *in vivo* via p53-dependent cellular arrest that probably is also associated with a perturbation of ribosome biogenesis. Presumably, these findings support the idea of a cross talking between rLZ-8 and the S7-MDM2-p53 pathway, providing a novel molecular mechanism whereby rLZ-8 may exerts its anticancer function, or at less in part. (118)

A similar effect against the proliferation of lung cancer cells was demonstrated using FIP-gts isolated from *Ganoderma tsugae* (64). Doses of 0.3 μM of rFIP-gts reduced the number of A549 cell culture colonies formed in agar by 50% when compared with control cells (119). In addition, an *in vivo* anti-tumor experiment was performed using nude mice xenograft model

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subcutaneously inoculated with A549 cells, showing a significant inhibition of tumor growth in mice treated with rFIP-gts at therapeutic doses of 12.8 mg/kg of body weight (119) Thus, this study has shown that rFIP-gts suppresses tumor growth *in vitro* and *in vivo*.

A similar effect against lung cancer was discovered in FIP-gmi, which exhibit 83.8% amino acids sequence similarity with FIP-gts (120). FIP-gmi shows a potent inhibitory effect on epidermal growth factor (EGF) when the peptide was cloned from *Ganoderma Microsporu*m and used as bioactive compound in cell culture of a lung cancer model (120). The EGF/EGFR interaction is important for the development of lung cancer, as it is well known that EGF induce invasion and metastasis of A549 lung cancer cells, leading to an increase of PI3K/Akt, which results in up-regulation of Rac1/Cdc42 activity and assembly of cell-cell contacts, as well as enhanced invasion by tumor cells (121-123). At this respect, it has been evidenced that FIP-gmi inhibits EGF-induced A549 cell migration and invasion by wound-healing assay and modified Boyden chamber via down-regulation of EGFR activity. This FIP down-regulated p-EGFR expression and inhibited cell migration and invasion of A549 model cells, through blocking of the EGF/phosphoEGFRPI3K/Akt pathway. In addition, significant changes were noticed in acting conformation following FIP-gmi repression of invasive growth (120) Thus, GMI might be an excellent candidate for use in clinical therapies and has considerable potential for lung cancer chemoprevention specifically.

There is another interesting drug therapy target where FIPs may have a significant therapeutic potential. For instance, because of the critical role of the nuclear transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) in inflammation, viral replication, carcinogenesis, antiapoptosis, invasion, and metastasis, specific inhibitors of this nuclear factor are being sought and tested as possible treatments for these diseases (124). NF-κB has been shown to regulate the expression of several genes, the products of which are involved in lung inflammation and tumorigenesis, including matrix metalloproteinase-9 (MMP-9). In addition, NF-κB constitutive activation is related to the progression of various malignant neoplasms by carcinogens, inflammatory agents, and tumor promoters, such as cigarette smoke, H_2O_2, and TNF-α (124).

The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα, a specific inhibitor of κB with sequence rich in ankyrin repeats, and phosphorylation of p65, a nuclear factor that in humans is encoded by the RELA gene. This in turn leads to the translocation of NF-κB from the cytoplasm to the nucleus, where it binds to specific response elements in the DNA. IκBα is one member of a family of cellular proteins that function to inhibit the NF-κB transcription factor; the IκBα protein sequesters the transcription factor NF-κB, maintaining it as an inactive complex in the cytoplasm (125).

It has been demonstrated that FIP-gmi from *Ganoderma Microsporum* suppresses the proteolytic activity of MMP-9 (126). In this research, using human alveolar epithelial A549 cells as a model. It demonstrated that MMP-9 activity induced by TNF-α is successfully interfered by FIP-gmi through down-regulation of NF-κB, probably by inhibiting its translocation. Apparently, the mechanism of bioactivity of this FIP involves interference with the inhibitory nuclear factor IκBα. In addition, these authors provide evidence that the phosphorylation, degradation, suppression, and translocation to the nucleus of p65 are also affected by FIP-gmi. Based on immunocytochemistry analysis, FIP-gmi suppressed TNF-α-induced nuclear translocation of NF-κB p65, impeding expression of MMP-9. When the expression of MMP-9 is increased constitutively, it raises the probability of metastasis in various cancers, including lung cancer. These findings support the importance of FIP-gmi in suppression of invasive activity through the NF-κB/MMP-9 pathway; therefore, FIP-gmi can be a potential chemo-preventive agent against invasion of tumor cells (126)

There are another molecular and cellular mechanisms related with cell pathological proliferation that have interesting potential for FIPs to be interfered, as it is suggested by recent experimental evidence. Autophagy is a lysosomal degradation pathway that controls the quality and balance of cytoplasmic constituents, involving protein and organelles; it is proposed as an important target for cancer therapy, and its disruption is a promising strategy for increasing sensitivity to therapeutic agents (127). It has been suggested that lysosome inhibitors enhance the effects of FIP-gmi on cell death, where this FIP acts as a mediator that increase the effects on autophagic cell death during co-administration with lysosome inhibitors bafilomycin-A1 and chloroquine (128). In that study, both lysosome inhibitors significantly enhanced FIP-induced cell death when FIP-gmi and bafilomycin-A1 are co-administrated at concentrations as low as 0.3 μM and 0.5 nM, respectively. This co-treatment induces the accumulation of large amounts of autophagosomes, but do not significantly induce apoptosis. FIP-gmi elicited autophagy through the PI3K/Akt/mTOR mechanism, a mammalian target of rapamycin signaling pathway. This mechanism is important because is related to drug resistance in some cancer therapy schemes; inhibition of lysosomes fusion results in accumulation of autophagosomes as an initial mechanism of cell death. Therefore, the induction of FIP-gmi mediated autophagy can be an interesting therapeutic option in the fight against some

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types of carcinogenic processes, including lung and colon cancer (61, 128).

Apparently, FIP-gmi also has the ability to stimulate apoptotic autophagy through caspase-7 pathway when combined with low-dose of cisplatin (a drug used in chemotherapy). The combination of 1.2 μM of FIP-gmi and 2.5 μM of cisplatin can increase the efficiency of the medication and reduce side effects observed when this drug is used at high doses (129). In addition, FIP-gmi elevates the intracellular calcium levels and reduces the growth of multidrug resistance sublines via autophagy and apoptosis, throughout the promotion of a significant pro-death and autophagy effect in multidrug resistant lung cancer cells, providing a potential strategy for overcoming multidrug resistance in the treatment of lung cancer (61).

FIPs also have exhibited antimetastasis properties during experimental assays. The formation of the centralspindlin complex is an important step necessary for several cellular functions that involve membrane deformations and cell movements, including metastasis, which makes of this process an important therapeutic target against malignant cells metastatic behavior. RacGAP1 is a gene that encodes a GTPase-activating protein (GAP) that is a component of the centralspindlin complex. It serves as a microtubule-dependent and Rho-mediated signaling required for the myosin contractile ring formation during the cell cycle cytokinesis. This protein binds activated forms of Rho GTPases and stimulates GTP hydrolysis, which results in negative regulation of Rho-mediated signals. This protein plays a regulatory role in cytokinesis, cell growth, and differentiation. Recently, a demonstration that RACGAP1-STAT3-survivin signaling pathway is required for the invasive phenotype of uterine carcinogenesis and is a newly identified therapeutic target in this lethal disease (130).

With respect to fungal immunomodulatory proteins, experiments demonstrate that oral administration of FIP-fve generates significant antitumor activity in mice bearing BNL hepatoma cells (131). In this study, FIP-fve treatment in combination with anti-IFN-γ neutralizes mAb, resulting in lower antitumor effect than with FIP-fve treatment alone, providing evidence that FIP-fve reduces RacGAP1 expression resulting in antimetastasis and suppression of the proliferation of A549 cells via p53 activation pathway. It was also shown that FIP-fve increases p53 expression, as well as the expression of its downstream gene p21, on a Western blot analysis. In addition, FIP-fve inhibited migration of A549 cells on wound healing assay and decreased filopodia fiber formation on labeling with Texas Red-X phalloidin. At FIP-fve concentrations of 3.75 and 7.50 μM, percentages of migrating cells decreased to 43percent and 14% at 24 h and 13 % and 3 % at 48 h, respectively, showed that FIP-fve induces dose-dependent decreases in both migration and invasion (131).

In other work, Xu *et al*. (2016) reported that, at a concentration of 10 μg/mL, FIP-gta from *Ganoderma atrum* could induce apoptosis and growth inhibition on cells MDA-MB-231, a cell line of breast cancer. The experiment suggest that FIP-gas could arrest the cell cycle in stage G1, because it was observed that a high percentage of cells were in this phase and a low percentage in S phase; Also, it was evident that the cells in the presence of FIP-gas had a high proportion in late apoptotic state, compared to control cells. Furthermore, this study analyzed the gene profile expression using microarrays and identified 669 differentially expressed genes, the grouping of these genes revealed that 96 are related to biological processes including cell death, cell growth and cell adhesion, among others. The authors performed real-time PCR to ten of these genes (TNFSF8, SQSTM1, DUSP1, SMPD1, BCL-2, JAK2, ITPR1, CCR10, Cckbr, and DRD1) whose function is related to cell growth and death; of these, only Cckbr was not overexpressed. The expression profile allowed to infer a possible mechanism of biological action generated by FIP-gas are by inhibiting cell growth and induce apoptosis in this cell line (132).

Findings, altogether, are an important contribution to understanding the impact of FIP on tumor cells. The elucidation of their possible molecular and cellular mechanisms of action is a step forward to the development of more effective cancer treatments.

8. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Purification of FIPs from wild or cultivated mushrooms remains low yielded, complicated, time consuming, and costly, so research is now focused on identifying new FIPs by other molecular methods and characterizing their expression. These methods include genomic and transcriptomic sequencing.

With the goal to obtain enough amounts of FIPs for practical uses, several genetic engineering approaches have been applied in order to improve the yields of peptide in cell culture. So far, the most developed expression systems are using *E. coli*, yeast and insect cells. The gene encoding FIPs has been effectively expressed in these prokaryotes and eukaryotes expression models. The FIP recombinant proteins produced in *E. coli*, with a tag consisting in an expressed fusion protein of glutathione S-transferase (GST), are soluble and high quantity, which can be convenient for their future industrial applications.

Nowadays, the yeast and insect cell expression systems are commonly used for the FIPs

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production, with the purpose to obtain proper folding and post-translational modifications. Yet, there is not consensus about these protein modifications to be necessary for the bioactivity in the vertebrate; in addition, these two expression systems are also facing disadvantages of long growth cycle, high cost, and complicated purification procedures.

The studies conducted on FIPs are commonly aimed to discovery of the characteristics and pinpointing the molecular pathways interfered in vertebrates by these proteins and exploring their potential in human health. Even though this goal is in the an early stage, there are still some important insights to understand some mechanisms of the FIPs activity, particularly in relation to its immunomodulatory effects on the vertebrate's immune system and as anti-cancer agents acting against malignant cells.

In spite of several researches demonstrating their potential benefits in the battle against several diseases or as adjuvants in the treatment of some important diseases, including cancer, inflammatory, allergic and immune deficiencies, there is still a need for more intensive research that convincingly support the development of a FIP-based new therapeutic drug. This underscore the importance of continuing the research on FIPs in order, not just to find new applications from promising mushroom species of great potential in human health, but to elucidate the complex molecular and cellular mechanisms and immunological pathways undergoing their modulatory effects.

Since the discovery in 1989 of the first fungal immunomodulatory protein (FIP-LZ8) from *Ganoderma lucidum*, there have gradually increased the reports of such proteins in different species of edible and medicinal mushrooms. Most studies focus on the assessment and clarification of cellular and molecular mechanisms triggered by this type of proteins because of their great potential in the activation of the immune response in human cells. In this context, OMIC sciences play a key role in the knowledge of these mechanisms so far poorly understood.

Regardless of being highly conserved in primary sequence and structure, the studies shown that FIPs family members have a broad spectrum of biological activities in vertebrate experimental models (cell culture and animals). The effects include systemic anaphylactic reactions, cell aggregation, hemagglutination, stimulation of mouse splenocytes proliferation and human peripheral blood lymphocytes, changes in the expression of several cytokines, particularly IL-2, IFN-γ, and TNF-α.

Considering all the evidence available so far, it can be stablished that FIPs appear as promising targets for the development of new therapeutic drugs for the treatment of various human diseases. They offer unique opportunities derived from its nature, as they are phylogenetically related with vertebrate's immune system, reflected mainly by its similarity with immunoglobulins. In the other hand, its small molecular size and not need of sophisticated post-translational peptide folding or biochemical modifications, make FIPs excellent candidates for manipulations by means of molecular engineering techniques and designing of scalable industrial production protocols.

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