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Expression and activity of a recombinant chimeric protein composed of pokeweed antiviral protein and of human interleukin-2

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Abstract The pokeweed antiviral protein (PAP) has already been used to chemically construct immunotoxins. Here we tested the recombinant approach for the production of PAP-containing cytotoxic fusion-proteins. A cDNA encoding a mutated PAP (PAP9), which is expressed at high levels in bacteria, was fused to human interleukin-2 (IL-2) cDNA. The resulting PAP9–IL-2 protein was as active as the free PAP9 in inhibiting an eukaryotic cell-free translation system. Only the chimeric protein desaminated the 28S rRNA and inhibited translation of the CTLL-2 cell line which expresses the IL-2 receptor. These results show that PAP is a suitable toxin for the production of recombinant immunotoxins.

Key words: Pokeweed antiviral protein; Recombinant cytotoxic fusion protein; Inhibition of translation; *N*-glycosidase activity

1. Introduction

The pokeweed antiviral protein (PAP), like other *N*-glycosidases, inactivates eukaryotic ribosomes and therefore inhibits the protein-synthesis process. Ribosome inactivation has been correlated with removal of one adenine residue of the large ribosomal RNA (rRNA). This adenine is located in an evolutionary conserved region and corresponds to position 4324 for rat liver 28S rRNA [1]. The ribosomes of *Phytolacca americana*, the PAP-producing plant, are also susceptible to PAP depurination [2].

The sequences of different forms of PAP produced by *P. americana* have been determined at the cDNA or protein levels [3–6]. The 3-dimensional structure has been established for PAP by X-ray crystallography to 2.5 Å resolution [7] and compared to that of the A-chain of ricin [8] for which key amino acids have been identified [9].

PAP has been chemically linked to antibodies in order to specifically kill cells expressing the epitope recognized by the antibody part of the resulting immunotoxins, and clinical trials have been performed with a potent immunotoxin (B43-PAP) against B-lineage leukemia/lymphoma cells [10–13]. In addition, PAP appeared to be a suitable ribosome-inactivating protein for the production of recombinant cytotoxic fusion proteins [14]. Here we produced and tested the cytotoxic activity of a recombinant hybrid protein composed of human interleukin-2 (IL-2) and a mutated form of PAP (PAP9) which allows *Escherichia coli* growth and is expressed at high levels in bacteria [15].

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2. Materials and methods

2.1. Construction and expression

The pBluescript KS vector digested with *Kpn*I and *Hin*dIII was ligated with both (i) the IL-2 insert of BBG3 (R and D Systems, Europe) digested with *Xho*I, treated with the Klenow DNA polymerase and digested with *Hin*dIII and (ii) the insert of the pBluescript KS clone of PAP9 digested with *Acc*I, treated with the Klenow DNA polymerase and digested with *Kpn*I. The insert of a positive clone was then transfered into the pKK233.2 expression vector by using *Nco*I and *Hin*dIII enzymes. The first three N-terminal amino acids of IL-2 have been deleted during the construction.

The proteins were expressed in *E. coli* and the inclusion body fraction isolated, solubilized and renatured by dialysis as previously described for PAP [15]. The preparations were analyzed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue to estimate the relative amounts of recovered recombinant proteins.

2.2. In vitro activity

The activity of the recombinant proteins was tested in vitro using the reticulocyte protein synthesizing kit type II (Boehringer) as previously described [15].

2.3. Cytotoxicity

The CTLL-2 cell line was maintained in RPMI-8866 medium complemented with 20% inactivated fetal calf serum, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M β-mercaptoethanol and 0.4 ng/ml IL-2 in humidified air at 37°C and 5% CO_2. The cells were washed twice in the same medium but without IL-2 and seeded in a 96-well plate at 2×10^4 cells/ml, to which the protein preparations were added. The cells were incubated 1 h at 4°C, then washed and incubated 45 h at 37°C in a medium containing IL-2. The medium was then replaced by a methionine-free medium and 30 min later 10 μCi of [^{35}S]methionine (Amersham) was added. After 2 h at 37°C, the cells were lyzed with 0.1 M NaOH, the cell proteins precipitated with trichloracetic acid on glass-fiber filters, and the incorporation measured by liquid scintillation counting (microbeta 1450 Wallac). The experiments were carried out in triplicate.

2.4. Analysis of 28S rRNA desamination

Desamination of 28S rRNA was analyzed by primer extension. The reaction was performed as described by Dore et al. [16] with RNA extracted from CTLL-2 murine cells. The oligodeoxynucleotide used is complementary with 28S rRNA from T4334 to T4350, a region 3′ to position 4256 corresponding to the PAP desamination site. Reverse transcriptase stops at this position yielding a 94-nucleotide-long cDNA if adenine is absent.

3. Results and discussion

3.1. Construction and expression

PAP alone has a very low toxicity for eukaryotic cells, but conjugates made with PAP and a monoclonal antibody (mAb) are highly cytotoxic immunotoxins for cells bearing the mAb-epitope [10–13]. In addition, PAP appeared to be a suitable ribosome-inactivating protein for the production of recombinant immunotoxins since an immunotoxin made with PAP remains cytotoxic when the linker used to bind the two pro-

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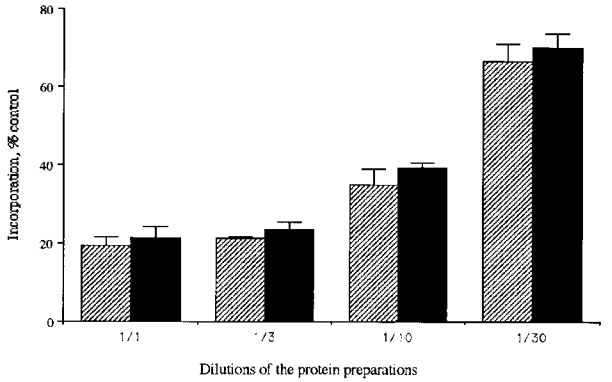

Fig. 1. Inhibition of a cell-free reticulocyte protein synthesis system. The level of incorporation of [^{35}S]methionine into TCA-precipitable products was determined as percentage of the incorporation obtained with a buffer-treated cell-free system. Analysis was done with PAP9 (shaded boxes) and PAP–IL-2 (black boxes) protein preparations.

teins together is a non-cleavable agent [14]. This property is not shared by all ribosome-inactivating proteins [14].

To study the feasibility of this approach, a construction was made by fusing PAP to human IL-2. A clone coding for a mutated form of PAP, PAP9, which permits *E. coli* growth and is expressed at high levels in bacteria was used for the construction [15]. Since fusions made to the N-terminus of other *N*-glycosidases reduced their activity [17,18], we choose to introduce IL-2 at the C-terminus of PAP9.

P. americana plants very likely produce a PAP precursor since the protein isolated from the leaves of the plant does not contain the N- and C-terminal regions encoded by the cDNA. Our PAP9 clone encodes a protein containing the C-terminal extension of the putative precursor. The C-terminal extension being dispensable for the *N*-glycosidase activity, the unique *Acc*I restriction site present in the middle of this region was used to make the PAP9–IL-2 construct. The deduced amino acid sequence present between PAP9 and IL-2 and which corresponds to the remaining part of the C-terminal extension of PAP9 is: Y, N, Q, N, A, M, F, P, Q, L, I, M.

E. coli cells tranformed with the construct were induced for expression. The inclusion body fraction, mainly containing the recombinant protein, was isolated, and a denaturation/renaturation process was used to prepare the soluble protein.

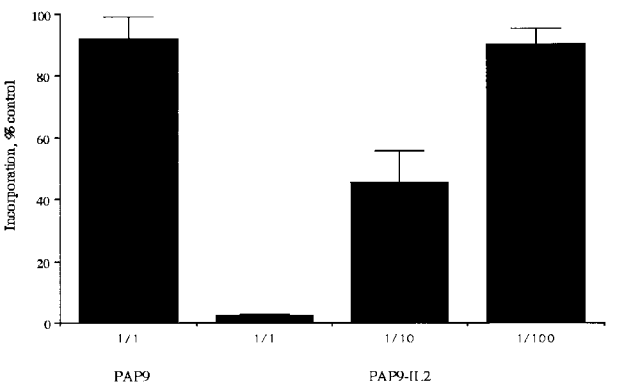

Fig. 2. Activity of recombinant proteins on the protein synthesis of CTLL-2 cell line. Incorporation of [^{35}S]methionine into TCA-precipitable products was referred to a positive control corresponding to buffer-treated cells. The cells were incubated with PAP9 or PAP9–IL-2 protein preparations. Different dilutions of PAP9–IL-2 protein preparation were also tested.

3.2. *In vitro PAP9 activity*

The PAP9 activity of the chimeric protein was tested in a cell-free protein-synthesizing system and compared to that of the non-fused PAP9 protein (Fig. 1). The results showed that PAP9–IL-2 was as active as the non-fused PAP9 protein in inhibiting eukaryotic translation. Therefore PAP9 activity was not affected by the presence of the ligand.

3.3. *Cytotoxicity of PAP9–IL-2*

The biological activity of PAP9–IL-2 was tested using the CTLL-2 cell line. This murine cell line requires IL-2 to proliferate and therefore expresses the IL-2 receptor. Since PAP9 interrupts translation, cytotoxicity was followed by measuring the level of protein synthesis in the cells. The cells were preincubated with PAP9 or PAP9–IL-2 protein preparations in a IL-2-free medium and then incubated in the medium containing IL-2. The results showed that PAP9–IL-2 inhibited cellular translation and that free PAP9 had no effect (Fig. 2). The level of inhibition was dependent on the amount of PAP9–IL-2 protein added to the cells (Fig. 2).

PAP inactivates ribosomes and therefore inhibits translation, its *N*-glycosidase activity removing one adenine residue of the 28S rRNA. This adenine, located in an evolutionary conserved sequence, corresponds to position 4256 for mouse 28S rRNA. To confirm that PAP9 activity of the hybrid protein was responsible for the cytotoxicity, after treatment we analyzed the integrity of 28S rRNA by primer extension (Fig. 3). An oligodeoxynucleotide hybridizing with a region 3′ to the targeted adenine was used as primer. The reverse transcription reaction stops if a base is missing on the matrix RNA yielding, in our case, a 94-nucleotide-long cDNA if A4256 is absent. The 28S rRNA extracted from cells treated with PAP9–IL-2 (lane 3) was indeed desaminated. Such desamination was neither observed with mock-treated (lane 1) nor with cells treated with PAP9 (lane 2). Inhibition of the translation correlated with the appearance of desaminated 28S

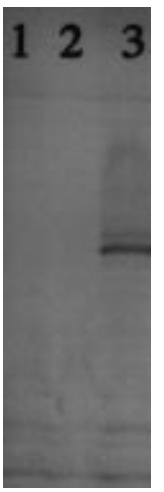

Fig. 3. Desamination analysis. Primer extensions were performed with RNAs extracted from mock-treated cells (lane 1), cells treated with PAP9 (lane 2) or with PAP9–IL-2 (lane 3) protein preparations. The specific cDNA, appearing if 28S rRNA had been desaminated by PAP activity, is visible in the middle of the figure.

rRNAs shows that PAP9 activity has been transported to the cytoplasm. Therefore PAP appeared to be a suitable ribosome-inactivating protein for the production of recombinant cytotoxic hybrid proteins, which are well-defined products.

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