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Production of recombinant anthrax toxin receptor (ATR/CMG2) fused with human Fc *in planta*

V. Andrianov [1], R. Brodzik [1], S. Spitsin, K. Bandurska, H. McManus, H. Koprowski, M. Golovkin *

Biotechnology Foundation Laboratories at Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA

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ABSTRACT

Mass vaccination against anthrax with existing vaccines is costly and unsafe due to potential side effects. For post-infection treatment, passive immunotherapy measures are currently available, most based on anthrax protective antigen (PA)-specific therapeutic antibodies. Efficient against wild-type strains, these treatment(s) might fail to protect against infections caused by genetically engineered *Bacillus anthracis* strains. A recent discovery revealed that the von Willebrand factor A (VWA) domain of human capillary morphogenesis protein 2 (CMG2) is an exceptionally effective anthrax toxin receptor (ATR) proficient in helping to resolve this issue.

Here we describe *in planta* production of chimeric recombinant protein (immunoadhesin) comprised of functional ATR domain fused with the human immunoglobulin Fc fragment (pATR-Fc). The fusion design allowed us to obtain pATR-Fc in plant green tissues in a soluble form making it fairly easy to purify by Protein-A chromatography. Standardized pATR-Fc preparations (purity > 90%) were shown to efficiently bind anthrax PA as demonstrated by ELISA and Western blot analysis. Recombinant pATR-Fc was also shown to protect J774A1 macrophage cells against the anthrax toxin. This study confirmed that plant-derived pATR-Fc antibody-like protein is a prospective candidate for anthrax immunotherapy.

Introduction

The occurrence of dangerous side effects associated with the current anthrax vaccine is halting mass vaccination efforts [1]. The development of preventive and/or post-exposure treatments has become urgent in view of the threat of bio-terrorism [2].

The search for efficient immunotherapy has confirmed that many molecules, peptides, and other compounds are able to block *Bacillus anthracis* toxins [3–6]. The PA-specific[2] antibodies that would be excellent for anthrax prophylaxis [7,8] might not be effective when used against genetically altered strains [9].

The recently discovered VWA domain of either TEM8 or the CMG2 receptors was shown to bind PA very efficiently [10] and was therefore capable of protecting cells against toxicity *in vitro* [11,12]. The soluble receptor decoy consisting of CMG2 domain (sCMG2) was able to protect animals against a lethal toxin challenge *in vivo* [13,14].

The main advantages of plants for use as bioreactors are their low-cost and a greater potential for scalability compared to microbial or animal systems [15,16]. An additional advantage, from the public health point of view, is greater safety, since plants do not contain mammalian pathogens.

Here we report the plant-based production of a potent ATR-based agent, the immunoadhesin, comprised of the VWA domain of CMG2 fused with human antibody Fc part fragment (pATR-Fc). The fusion of heterologous peptides with Fc is known to provide better yields, stability and facilitate purification process in different systems, including plants [17–21]. The retention of this recombinant protein in the endoplasmic reticulum (ER) has allowed for its abundant expression and accumulation in plants without any adverse effects and being mostly in soluble form makes it feasible for simple protein-A-based purification. *In vitro* characterization of pATR-Fc preparations (purity > 90%) confirmed that it binds to commercial PA and is able to protect J774A1 macrophage cells against challenge with anthrax toxin. At this point, the study suggests pATR-Fc as a functional recombinant plant-based therapeutic agent suitable for post-infection therapy of anthrax.

Materials and methods

Genetic engineering of recombinant plant codon-optimized ATR-Fc fusion

The 171 amino acid part of CMG2 protein (VWA domain, aa 44–214, Accession Number pdb|1TZN|AA) was selected for stable plant-based expression as part of a chimeric Fc fusion recombinant

* Corresponding author. Fax: +1 215 503 6795.
E-mail address: maxim.golovkin@jefferson.edu (M. Golovkin).

[1] Two authors contributed equally.

[2] *Abbreviations used:* PA, protective antigen; ATR/CMG2, anthrax toxin receptor/capillary morphogenesis protein 2; VWA, the von Willebrand factor A; Fc, constant fragment of human immunoglobulin.

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protein. The plant codon-optimized DNA fragment of 513 bp was synthesized using two-step PCR where the first primer-extension reaction was performed at 35 repetitions using the following cycle: 94°, 20 s/50°, 20 s/68°, 60 s with 2 μM of each of the following overlapping oligonucleotides [Operon, Huntsville, AL]:

5′-TGG ATC TTT ATT TTG TTC TTG ATA AAT CTG GTT CTG TTG CTA ATA ATT GGA TTG AAA TTT ATA ATT TTG T-3′
5′-TAA AAG AAA GTC TCA TTT CAG GAG AAA CAA ATC TTT CAG CAA GTT GTT GAA CAA AAT TAT AAA TTT CAA T-3′
5′-TGA AAT GAG ACT TTC TTT TAT TGT TTT TTC TTC TCA AGC TAC TAT TAT TCT TCC TCT TAC TGG TGA TAG A-3′
5′-CCA ACA GGA GAA ACT CTT TTC AAA TCC TCA AGT CCT TTA GAA ATT TTT CCT CTA TCA CCA GTA AGA GGA A-3′
5′-AAA AGA GTT TCT CCT GTT GGT GAA ACT TAT ATT CAT GAG GGA CTT AAA CTT GCT AAT GAA CAA ATT CAA A-3′
5′-TCC ATC AGT AAG AGC AAT AAT AAT AGA AGA AGT TTT AAG ACC ACC AGC TTT TTG AAT TTG TTC ATT AGC A-3′
5′-TTA TTG CTC TTA CTG ATG GAA AAC TTG ATG GTC TTG TTC CTT CTT ATG CTG AAA AAG AAG CTA AAA TTT C-3′
5′-GTT CAA AAT CAA GAA CAC CAA CAC AAT AAA CAG AAG CAC CAA GTG ATC TTG AAA TTT TAG CTT CTT TTT C-3′
5′-TGG TGT TCT TGA TTT TGA ACA AGC TCA ACT TGA AAG AAT TGC TGA TTC TAA AGA ACA AGT TTT TCC TGT T-3′
5′-GCC GCA AGA ATA GAA TTA ATA ATT CCT TTA AGA GCT TGA AAT CCA CCC TTA ACA GGA AAA ACT T-3′

The resulting product was diluted 1:1000 and amplified using the regular PCR reaction with the following primers: F: 5′-AGT CCC ATG GAT CTT TAT TTT GTT CTT-3′ and R: 5′-TCC TGC GGC CGC AAG AAT AGA ATT AAT-3′. The fragment was gel extracted and sub-cloned into *p1.2* Impact Vector [Plant Research International, Wageningen, The Netherlands] using NcoI–NotI sites. After sequence verification, the fragment was placed under the Rubisco promoter as a fusion with human Fc using a flexible hinge [21] and carrying an apoplastic (Apo) signal, with or without ER C-terminal <KDEL>; both expression cassettes contained 6× His and c-*myc* detection/affinity-purification tags (Fig. 1). For plant transformation, the expression cassettes were further sub-cloned (PacI–AscI) into the binary *pBIN-PLUS* vector (*pATR-Fc*$_{Apo}$ and *pATR-Fc*$_{ER}$) and put in *Agrobacterium tumefaciens* LBA4404 strain suitable for stable tobacco plant transformation targeting to apoplastic region or ER, respectively.

Plant transformation

A stable agrobacterium-mediated transformation procedure was performed according to standard protocol [22]. Transgenic tobacco lines (cv Wisconsin 38) were selected using kanamycin at a final concentration of 100 mg/l. Rooted shoots were then transferred to soil and grown to maturity in greenhouse conditions.

Isolation and purification of recombinant proteins

Total and soluble plant proteins were isolated as described previously with minor modifications [23]. Frozen tobacco plant leaves were homogenized in PBS buffer containing 0.5% Tween-20 and clarified by centrifugation at 15,000*g* for 30 min. The supernatant was filtered through a miracloth [Calbiochem, La Jolla, CA] applied to a protein-A column [Amersham, Piscataway, NJ] and pATR-Fc was eluted according to the manufacturer's recommendations. After overnight dialysis against PBS, pATR-Fc was concentrated to 1.45 mg/ml using an Amicon Ultra spin-column with a 35-kDa cut-off [Millipore, Bedford, MA] and stored at −80 °C. [21,24].

Protein analysis

In vitro characterization and quantification of protein expression was performed with ELISA and Western blot analysis essentially as described [21,25].

Affinity pull down assay

Commercial preparation (0.5 μg) of 83 kDa form of anthrax PA (PA_{83}) protein #171A from List Laboratories [Campbell, CA] was mixed with 10 μg pATR-Fc and brought to 100 μl with Tris buffered saline (TBS) plus 0.05% Tween-20 (TBST), 3% BSA, and 1 mM $MgCl_2$ for overnight incubation at 4 °C. Commercial anti-PA goat antiserum [List Laboratories] (10 μg) was used as positive control. Bound protein complexes were rescued from the solution using MagnaBind Protein-A Beads [Pierce, Rockford, IL] following the manufacturer's protocol with some minor modifications. Elu-

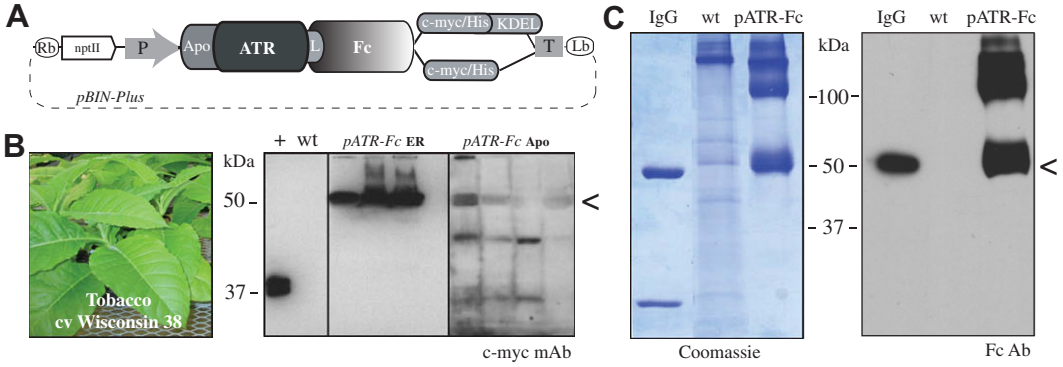

Fig. 1. Production of recombinant ATR-Fc protein in plants. (A) Schematic diagram of pATR-Fc expression cassette arrangements in the binary vector *pBIN-Plus* for *Agrobacterium*-mediated plant transformation, resulting in *pATR-Fc*$_{Apo}$ and *pATR-Fc*$_{ER}$ constructs. Synthetic cDNA fragment encoding CMG2 protein domain (ATR) fused with human IgG-Fc fragment via the flexible linker peptide (L) placed between the rubisco promoter (P) and terminator (T), with addition of N-terminal apoplastic signal (Apo) with or without C-terminal ER retention peptide <KDEL> and both having C-terminal c-*myc* and His tags. Both cassettes are placed in the transfer DNA region between the left (LB) and right (RB) borders that contain NPTII gene expressing cassette for kanamycin selection. (B) The presence of pATR-Fc protein in extracts of transgenic tobacco plants of cv Wisconsin 38 (left panel) was confirmed by Western blot analysis (reducing conditions) using anti-c-*myc* monoclonal antibodies (right panel). Samples are shown for the constructs carrying ER retention signal (*pATR-Fc*$_{ER}$) or apoplast signal (*pATR-Fc*$_{Apo}$). *E. coli* expressed foreign peptide with c-*myc* tag used as a positive control (+). WT indicate the protein sample from wild-type tobacco. Molecular weight measured in kilodaltons (kDa). (C) The purified preparation of pATR-Fc was tested on SDS–PAGE at reducing conditions and either visualized by Coomassie blue staining (left), or tested with human Fc-specific antibodies on corresponding (diluted 1:10) Western blot (right). The band of expected molecular weight (47.7 kDa) of pATR-Fc is indicated by arrowhead. The extracts from wild-type tobacco (WT) and the human IgG were used as corresponding negative and positive controls. Molecular weight measured in kilodaltons (kDa).