International Immunopharmacology 19 (2014) 110–118

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

PTD-mediated intracellular delivery of mutant NFAT minimum DNA binding domain inhibited the proliferation of T cells

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ARTICLE INFO

Article history:
Received 28 January 2013
Received in revised form 31 December 2013
Accepted 3 January 2014
Available online 16 January 2014

Keywords:
Immunosuppressant
PTD
NFAT
Mutant minimum DNA binding domain of NFAT1
IL-2

ABSTRACT

The nuclear factor of activated T cell (NFAT) family of calcium-regulated transcription factors plays a key role in the development and function of the immune system. Calcineurin, a protein phosphatase, activates NFAT by dephosphorylation. The activated NFAT is translocated into the nucleus, where it up-regulates the expression of interleukin 2 (IL-2) and other target genes. Calcineurin inhibitors such as cyclosporine A (CsA) and FK506 are effective immunosuppressant drugs and dramatically increase the success rate of organ transplantation procedures. However, since calcineurin is expressed in most tissues in the body and calcineurin inhibition alters many cellular processes besides immune cell activation, the therapeutic use of calcineurin inhibitors is limited by serious side effects. Thus inhibiting NFAT by other mechanisms such as blocking its binding to DNA could be a more selective and safer approach to target NFAT for therapeutic applications. In peripheral T cells, productive immune responses are dependent upon the cooperative binding of the NFAT/AP-1 transcriptional complex to the promoter regions of genes such as interleukin-2 (IL-2), while NFAT in the absence of AP-1 leads to T cell anergy. Protein transduction domains (PTDs) are able to penetrate cell membranes and can be used to transport exogenous proteins across the cell and nuclear membranes. In this study, we constructed a fusion protein of PTD and a minimum DNA binding domain of human NFAT1 (PTD-ΔNFATminiDBD), which contains two mutations (R466A and T533G) in the AP-1 binding sites. The delivery and functions of this fusion protein in T cells were investigated. The results indicated that PTD-ΔNFATminiDBD could be effectively delivered into T cells and transported into the nucleus. PTD-ΔNFATminiDBD attenuated IL-2 production in T cells and then inhibited T cell proliferation, likely through competing against endogenous NFAT for binding to the IL-2 gene promoter. These results demonstrated that PTD-ΔNFATminiDBD was an effective NFAT inhibitor with a novel mechanism of action and might potentially be used as an immunosuppressant for organ transplantation with higher safety and better tolerance than calcineurin inhibitors.

1. Introduction

NFAT was first identified in T cells as an inducible nuclear factor able to bind to the distal antigen receptor responsive element of the human IL-2 promoter [1]. The NFAT family of transcription factors regulates the expression of various genes, including signaling proteins, cytokines, cell surface receptors, and cell cycle and apoptosis related proteins [2–4]. The NFAT family consists of four proteins that are regulated by the calcium/calcineurin-signaling pathway, known as NFAT1 (NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3) and another protein named NFAT5 (also called tonicity-responsive enhancer-binding protein, TonEBP), which is regulated by hyperosmotic stress [5–7]. Different NFAT transcription factors share high sequence similarity in the DNA binding domains (DBDs), which are distantly related to the DBDs of Rel/NF-κB. In resting T cells, NFAT proteins are phosphorylated, reside in the cytoplasm, and show low affinity for DNA. When T cells are stimulated by antigens, PHA, PMA and ionomycin, or other stimulators, NFAT proteins are rapidly dephosphorylated by calcineurin and translocated into the nucleus. The dephosphorylated (activated) NFAT proteins show increased affinity

Abbreviations: DBDs, DNA binding domains; ΔNFATminiDBD, a minimum DNA binding domain with point mutation in R466A and T533G of human NFAT1; eGFP, enhanced green fluorescent protein; NFATminiDBD, a wild type mini DNA binding domain of human NFAT1; NFAT, nuclear factor of activated T cell; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; PI, propidium iodide; PTD, protein transduction domain; QPCR, quantitative polymerase chain reaction; TonEBP, tonicity-responsive enhancer-binding-protein.

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http://dx.doi.org/10.1016/j.intimp.2014.01.001

for DNA binding [8,9]. AP-1 is induced by B7/CD28-mediated co-stimulatory signals in T cells, and productive immune responses are dependent upon the cooperative binding of the NFAT/AP-1 transcriptional complex to the promoter regions of certain genes [10]. These genes encode factors involved in the immune responses including IL-2, a major T cell growth factor, and IL-2 receptor α chain (CD25) [11]. However, a distinct transcriptional program of anergy executed by NFAT in the absence of AP-1 leads to the inability of T cells to mount a proliferative response to TCR engagement. Consequently, T cells fail to produce IL-2 or differentiate into effector cells to produce other cytokines such as IFN-γ or TNF-α [12]. In contrast to activated T cells, anergized T cells express increased levels of IL-10, a potent anti-inflammatory cytokine [13,14]. Cyclosporin A (CsA) and FK506 block calcineurin, a phosphatase that activates NFAT and resides in the upstream signaling of T cell activation [15]. Calcineurin has wide expression in most tissues in humans and is involved in many cellular functions in addition to T cell activation. Thus calcineurin inhibitors cause serious side effects that limit their therapeutic usage. Inhibitors that act on NFAT directly and do not target calcineurin phosphatase activity would inhibit T cell activation without toxicity like calcineurin inhibitors, and thus may be ideal immunosuppressive therapies [15,16]. Based on the crystal structure of NFAT/AP-1-DNA complex, amino acids from 391 to 583 of human NFAT1 constitute the minimum but sufficient domain for DNA and AP-1 binding, in which Arg 466 and Thr 533 are two key amino acids for NFAT/AP-1 binding but not important for NFAT/DNA binding [17,18]. Hence, we proposed that a minimum mutant DNA binding domain of NFAT1 with R466A and T533G substitutions (ΔNFATminiDBD) would impair affinity for AP-1 but retain high affinity for DNA binding, and this mutant would bind to DNA and then inhibit the endogenous NFAT/AP-1 complex binding. Meanwhile, since the mutant would not bind AP-1 and no genes undergo activation. Thus ΔNFATminiDBD could inhibit NFAT signaling downstream of calcineurin and may be a more selective and hypotoxic immunosuppressant than calcineurin inhibitors.

The protein transduction domains (PTDs), sometimes termed cell-penetrating peptides (CPPs), have been reported to efficiently translocate across the membrane of mammalian cells and mediate nuclear delivery of heterologous proteins fused to PTDs [19–21]. A significant number of biomacromolecules such as peptides, proteins, plasmid and nucleic acids have been reported to be transported into cytoplasm or nucleus via PTDs [20,21]. PTDs of membrane-permeable proteins and peptides include HIV-1 transactivator of transcription (TAT) protein [22–25], *Drosophila melanogaster* homeobox protein antennapedia [26,27] and herpes simplex virus protein VP22 [28,29]. The PTD of TAT, an 11aa peptide rich in arginine and lysine residues (YGRKKRRQRRR), was shown to be sufficient for the intracellular transportation and subcellular localization of proteins, and was proposed to be superior to other PTDs owing to its small size [23,30]. The HIV-1 TAT-PTD is becoming a useful molecular tool to enable the study of a broad spectrum of recombinant proteins for biotherapeutic applications. It also serves as a tool to facilitate the development of a new drug delivery platform.

In the present study, we constructed a fusion protein of TAT-PTD and ΔNFATminiDBD (PTD-ΔNFATminiDBD). We found that the fusion protein was rapidly and effectively delivered into the cytoplasm and nucleus of human T-lymphoma Jurkat cells in vitro. We then examined the potential immune suppressive function of PTD-ΔNFATminiDBD in transduced T cells in vitro. Our data demonstrated that PTD-ΔNFATminiDBD inhibited T cell proliferation and also attenuated their ability to produce IL-2 in vitro.

2. Materials and methods

2.1. Plasmids, chemical reagents and antibodies

pQE30 vector and *E. coli* strains DH5α, M15 were purchased from Qiagen China (Shanghai) Co., Ltd. (Shanghai, China). The plasmids of pMD18-T and pEGFP-N1; *Pfu* DNA polymerase; restriction enzymes of *KpnI*, *SalI* and *HindIII*; DNA Marker DL2000, λ-EcoT14 I digest DNA marker and unstained protein molecular weight marker were bought from Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). NFAT-AP1-IL-2-P/luciferase reporter vector and pRL-TK vector were kind gifts from Dr. Xiao He and Professor Yan Li [31] (Department of Molecular Immunology, Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing 100850, China). PVL-GST-NFATp was a kind gift from Professor James A. Goodrich (Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, CO 80309-0215, USA) and described previously [32]. Anti-6× His-Tag and anti-NFAT antibodies were purchased from Cell Signaling Technology, Inc (China) (Shanghai, China). Antibodies against Lamin B and β-actin were the products of Bioworld Technology Co., Ltd. (Minnesota, USA). Agarose was the product of Biowest (Madrid, Spain). Fermentas T4 DNA ligase was bought from Thermo Fisher Scientific (China) Co., Ltd. (Shanghai, China). Gel, PCR Clean-up system and Dual Luciferase Reporter System were from Promega (Beijing) Biotech Co., Ltd. (Beijing, China). Profinity IMAC Resins were purchased from Bio-Rad Laboratories Shanghai Ltd (Shanghai, China); IPTG was a product of Merck Millipore International (Darmstadt, Germany); GIBCO® Fetal bovine serum (FBS) and RMPI-1640 media were purchased from Invitrogen China Limited/Applied Biosystems China Limited (Shanghai, China). Goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (Philadelphia, USA). Amersham ECL Prime Western Blotting Detection Reagent from GE Healthcare Life Sciences (Shanghai, China); The *E. coli* culture media LB (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 10 g/L) were prepared as described elsewhere [33]. All other chemicals and reagents were of the highest analytical grade.

2.2. Methods

2.2.1. Construction of recombinant protein expression plasmids

All the expression plasmids were constructed as indicated in Fig. 1A. The NFATminiDBD from 391aa to 583aa of NFAT protein was amplified from the PVL-GST-NFATp plasmid and the details of the ΔNFATminiDBD construction, containing two point mutations T533G and R466A, have been shown in Supplemental Fig. 1. The ΔNFATminiDBD and/or eGFP sequences were inserted into the pQE30 or pQE30-PTD plasmid respectively. The expression vectors of pQE30-PTD-ΔNFATminiDBD-eGFP, pQE30-PTD-ΔNFATminiDBD and pQE30-ΔNFATminiDBD were constructed respectively and indicated in Supplemental Fig. 2. The recombinant plasmids were verified by PCR, restriction digestion and DNA sequencing assays (Fig. 1B). Primer synthesis and DNA sequence analysis were performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and the sequences of all PCR primer pairs are listed in Table 1.

2.2.2. Expression and purification of fusion proteins

The expression and purification of fusion proteins were performed as indicated previously [34]. Briefly, *E. coli* M15 cells were transformed with the identified recombination plasmids and induced with 1 mM IPTG for 6 h in LB. The expression levels of the fusion proteins were validated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis. Then *E. coli* M15 cells were sonicated in buffer A (containing 50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 7.9), and the supernatant was collected by centrifugation (12,000 *g*, 10 min) for purification and renaturation on Profinity IMAC Ni-Charged Resin column (Bio-Rad Laboratories Shanghai Ltd. (Shanghai, China)) according to the manufacturer's instructions. The purified proteins were identified by Western blotting with anti-6× His-Tag mAb. Endotoxins were removed with ToxinEraser™ endotoxin removal resin (GenScript USA Inc., NJ, USA), and protein concentration was estimated by Bradford method. Proteins were filtered through a 0.20 mm filter (Pall Corporation, MI, USA) and 0.25 mL aliquots were stored at −80 °C for later use.