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Use of genetic immunization to raise antibodies recognizing toxin-related cell surface ADP-ribosyltransferases in native conformation

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Abstract

ADP-ribosyltransferases (ARTs) transfer ADP-ribose from NAD to arginine, asparagine, or cysteine residues in target proteins. This post-translational protein modification is the mechanism by which cholera-toxin and other bacterial toxins cause pathology in human host cells. Molecular cloning has identified five toxin-related GPI-anchored cell surface ARTs in the mouse (ART1, ART2.1, ART2.2, ART3, and ART4) and three in the human (ART1, ART3, and ART4). ART2—which has sparked interest because of its ability to activate the cytolytic P2X7 purinergic receptor by ADP-ribosylation—is encoded by two functional gene copies in the mouse genome while the human genome carries two inactivated ART2 pseudogenes. We generated stable transfectants for FLAG-tagged versions of each of the functional human and mouse ARTs. Using genetic immunization we raised monoclonal antibodies that recognize the native human ARTs on the surface of living cells. Some of these mAbs recognize an epitope shared with the mouse ART orthologue but not with more distant ART paralogues. Screening of primary cells and established cell lines by FACS revealed expression of ART1 by monocytes, neutrophils and myeloid leukemia cell lines but not by cell lines derived from solid tumors. ART1 and ART4 have been assigned the designations: CD296, and CD297, respectively.

Keywords: Monoclonal antibodies; ADP-ribosylation; Genetic immunization; GPI-anchored molecule

1. Introduction

ADP-ribosyltransferases (ARTs) transfer ADP-ribose from NAD to arginine, asparagine, or cysteine residues in target proteins [1,2]. This posttranslational protein modification is the mechanism by which cholera- and other bacterial toxins cause pathology in human host cells [3] (Fig. 1). Molecular cloning has identified four toxin-related GPI-anchored mammalian cell surface ARTs (ART1–ART4) and one secretory ART (ART5) [4,5]. Mouse ART2 has sparked particular interest because of its ability to ADP-ribosylate and activate the cytolytic P2X7 purinoceptor, causing calcium-flux, shedding of CD62L, exposure of phosphatidylserine and cell death [6]. Intriguingly, the ART2 homologues of the human and chimpanzee are inactivated by premature nonsense mutations, while the mouse contains two closely related, functional ART2 genes [7,8].

The recent sequencing of the human and mouse genomes has uncovered the sequences of thousands of novel genes, to which antibodies are still lacking. Peptide and cDNA immunization are useful approaches for inducing highly specific antibodies against the predicted protein products. Anti-peptide antibodies are directed against linear epitopes and generally function well in Western blot analyses but often fail to recognize the antigen in native conformation. In contrast antibodies raised by cDNA immunization generally recognize the target antigen in native conformation because the antigen is synthesized and processed to its native conformation by the

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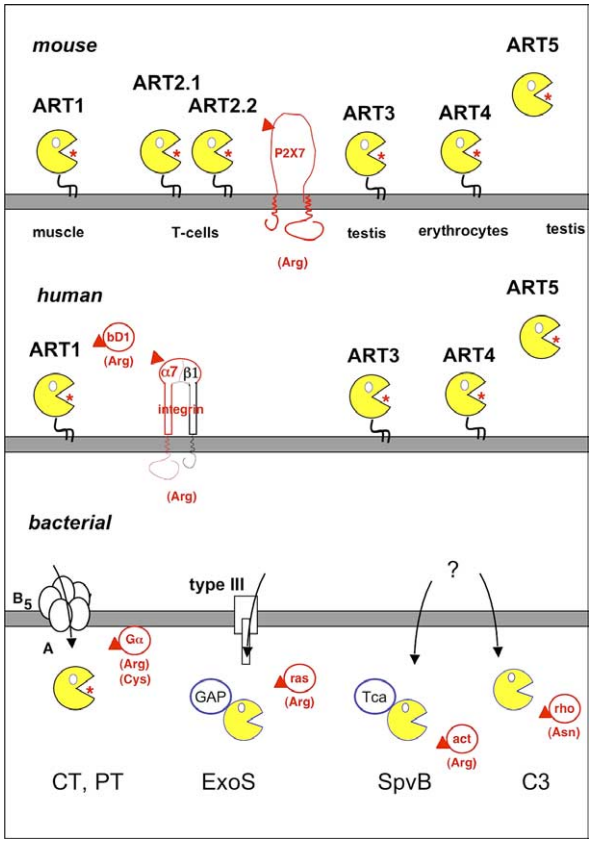

Fig. 1. Schematic diagram of the ART family. Molecular cloning has identified four toxin-related GPI-anchored mammalian cell surface ARTs (ART1–ART4) and one secretory ART (ART5). Mouse ART2 has sparked particular interest because of its ability to ADP-ribosylate and activate the cytolytic P2X7 purinoceptor. Intriguingly, the mouse contains two closely related, functional ART2 genes, while the ART2 orthologous gene of the human is inactivated by premature nonsense mutations. The primary sequence and 3D structure of ART2 show close structural similarity with a subgroup of bacterial toxins including the C3 exoenzymes of *Staphylococcus aureus* and *Clostridium botulinum*, exoenzyme S of *Pseudomonas aeruginosa* and SpvB of *Salmonella enterica.*

cells of the immunized host [9,10]. Here, we describe the use of the genetic immunization strategy to generate antibodies recognizing each of the GPI-anchored ecto-ADP-ribosyltransferases ART1–ART4 from mouse and man.

2. Materials and methods

2.1. Cloning of expression vectors, genetic immunizations, and selection of ART-specific mAbs

Expression constructs for epitope-tagged, GPI-anchored human and mouse ART1–ART4 were cloned by replacing the endogenous leader peptide with the leader of CD8a followed by a FLAG-tag [10] (Fig. 2). In case of ART2.2 an additional expression construct was cloned in which the endogenous GPI-signal peptide was replaced by the transmembrane and cytoplasmic domains of CD8a [11]. ART-expression constructs were conjugated to 1 μm gold particles (Bio-Rad) and rabbits and rats were immunized by ballistic DNA immunization (pressure setting at 400 ψ). Animals received four immuni-

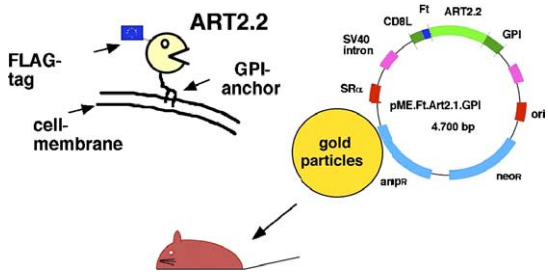

Fig. 2. Schematic diagram of the ART expression constructs and immunization strategy for generating ART-specific antibodies. Expression constructs for FLAG-tagged ARTs were cloned by replacing the endogenous leader peptide with the leader of CD8 followed by a FLAG-tag. These constructs were transfected into DC27.10 cells and stable transfectants were selected by cultivating cells in the presence of G418. ART-expression constructs were conjugated to 1 μm gold particles and rats and rabbits were immunized by ballistic DNA immunization.

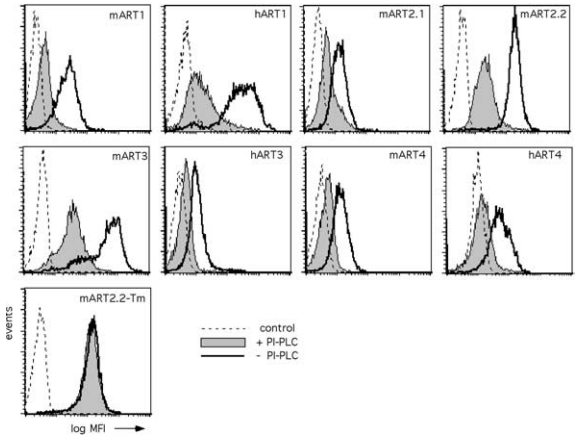

Fig. 3. FACS analyses of FLAG-tagged ARTs on the surface of stably transfected lymphoma cells. DC27.10 cells stably transfected with the indicated ARTs were incubated for 60 min in the presenced (shaded histograms) or absence (open histograms) of phosphatidyl inositol specific phospholipase C (PI-PLC) which cleaves GPI-anchored proteins from the cell surface. Cells were stained with saturating amounts of FITC-conjugated anti-FLAG mAb M2 and subjected to FACS analyses. Control stainings were performed using untransfected cells (dotted histograms) and cells transfected with mouse ART2.2 carrying the transmembrane domain of CD8a instead of the GPI-anchor (ART2.2-Tm). Histograms show the mean fluorescence intensities of vital cells-gated on the basis of exclusion of the DNA-staining dye propidium iodide.

zations in three-six week intervals with eight shots of plasmid-conjugated gold particles (1 μg DNA|mg gold|shot). Serum samples were obtained 10 days post immunization. For monoclonal antibody production, animals received a final boost with purified recombinant protein (50 μg in 200 μl PBS i.v.) three days prior to sacrifice. Splenocytes were fused to Sp2/0 myeloma cells and screened for production of ART-specific antibodies by ELISA using untransfected and ART-transfected cells as targets and alkaline phosphatase conjugated anti-rat IgG and OPD to detect bound antibodies. mAbs were isotyped using a rat immunoglobulin isotyping kit according to the manufacturer's (BD Bioscience) instructions. Antibodies were purified by affinity chromatography on Protein G–Sepharose (Pharmacia), and conjugated to Alexa488 according to the manufacturer's (Molecular Probes) instructions.