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PIN-bodies: A new class of antibody-like proteins with CD4 specificity derived from the protein inhibitor of neuronal nitric oxide synthase ☆

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Abstract

By inserting the CB1 paratope-derived peptide (PDP) from the anti-CD4 13B8.2 antibody binding pocket into each of the three exposed loops of the protein inhibitor of neuronal nitric oxide synthase (PIN), we have combined the anti-CD4 specificity of the selected PDP with the stability, ease of expression/purification, and the known molecular architecture of the phylogenetically well-conserved PIN scaffold protein. Such "PIN-bodies" were able to bind CD4 with a better affinity and specificity than the soluble PDP; additionally, in competitive ELISA experiments, CD4-specific PIN-bodies were more potent inhibitors of the binding of the parental recombinant antibody 13B8.2 to CD4 than the soluble PDP. The efficiency of CD4-specific CB1-inserted PIN-bodies was confirmed in biological assays where these constructs showed higher potencies to block antigen presentation by inhibition of IL-2 secretion and to inhibit the one-way and two-way mixed lymphocyte reactions, compared with soluble anti-CD4 PDP CB1. Insertion of the PDP into the first exposed loop (position 33/34) of PIN appeared to be the most promising scaffold. Taken together, our findings demonstrate that the PIN molecule is a suitable scaffold to expose new peptide loops and generate small artificial ligand-binding products with defined specificities.

Keywords: Scaffold protein; CD4; Protein inhibitor of neuronal nitric oxide synthase; Ligand binding; Paratope-derived peptide; Antibody

Creating small novel ligands with defined target specificity and biological properties which can mimic antibodies is one major goal of protein engineering. We and others have demonstrated that peptides derived from the antibody binding pocket, known as paratope-derived peptides (PDP), can achieve such objectives but also possess limitations [1]. The PDPs show considerable conformational flexibility in solution so that the entropic penalty upon binding is high, usually leading to significantly reduced affinities. Furthermore, their low proteolytic stability prevents or limits their use in biological assays and consequently in therapy [2,3]. To overcome such problems, one can chemically modify the PDPs through C-terminal amidation and N-terminal acetylation [4] or reduction of peptide bonds [5,6]. PDPs can be synthesized as retro-inverso peptides [7] or by using D-residues [8]. The flexibility and stability of peptides can also be restricted by grafting them onto a rigid scaffold

☆ *Abbreviations:* PIN, protein inhibitor of neuronal nitric oxide synthase; NO, nitric oxide; CDR, complementary-determining region; mAb, monoclonal antibody; H, variable region of the heavy chain; L, variable region of the light chain; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PDP, paratope-derived peptide; sCD4, soluble CD4; PBL, peripheral blood lymphocytes; MLR, mixed lymphocyte reaction; IL-2, interleukin-2.

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protein. Ideally, scaffold proteins should display several features like a phylogenetically well-conserved sequence among species in order to prevent or limit immunogenicity, or a robust architecture with a well-known three-dimensional molecular organization (crystallography or NMR available), a small size, no or only a low degree of post-translational modifications, and the fusion protein should be easily produced, expressed, and purified. More importantly, the scaffold protein must contain regions that can be extensively reshaped by residue insertion or deletion without affecting its overall folding and stability properties. Various scaffold proteins have been proposed up to date but none of them retains all the above properties [9].

The protein inhibitor of neuronal nitric oxide synthase (PIN) is a highly conserved protein of 89 amino acids, displaying 90% sequence identity between *Chlamydomonas*, *Caenorhabditis elegans*, *Drosophila*, and humans. PIN was originally identified as one of the light chains of flagellar and cytoplasmic dyneins and subsequently named LC8 (light chain of 8 kDa) [10,11]. PIN was also found to be a light chain for the unconventional myosin V [12,13]. Besides its involvement in two molecular motors, PIN was also demonstrated to interact with the N-terminal region of neuronal NO synthase (nNOS), thereby inhibiting nNOS dimerization and subsequent NO production [14]. A PIN protein from human origin, C-terminally tagged with a 6-histidine sequence, was successfully affinity purified from *Escherichia coli* transformed with the pET21b-PIN plasmid in our laboratory [15]. PIN is composed of a central C-terminal β sheet containing four anti-parallel β strands (β1, β4, β5, and β2) and two N-terminal antiparallel α helices (α1 and α2) on one side and a protruding β strand (β3) on the other side, as determined by NMR spectroscopy and X-ray crystallography [16–18]. The loop connecting the α1 and α2 helices (residues 30–35) and the two turns between the β2 and β3 strands (residues 60–62) and the β4 and β5 strands (residues 78–80) protrude on the same face outside the molecule and offer an interesting starting point for inserting PDPs of defined specificity.

The transmembrane glycoprotein CD4 is a major molecular partner in the immunological synapse which leads to the optimal activation of T lymphocytes during the immune response [19]. CD4 also serves as the primary receptor for the human immunodeficiency virus (HIV), thereby allowing the virus to enter cells [20]. The anti-CD4 chimeric recombinant antibody 13B8.2 was previously expressed in the baculovirus/insect cell system. We demonstrated in vitro that this antibody inhibited T lymphocyte IL-2 secretion [21] following antigen presentation and also prevents HIV transcription in $CD4^+$ cells at a post-gp120 binding step [21–23]. From this antibody, we have developed the concept of paratope-derived peptides (PDPs), corresponding to short amino acid sequences derived from antibody variable regions [2,24–26] which are screened from a systematic exploration of antibody variable domain sequences by the Spot method [27,28]. We then demonstrated that the bioactive PDP CB1, derived from the CDR1-H region of the anti-CD4 13B8.2 mAb, displayed biological properties similar to those of the parental 13B8.2 mAb but with the intrinsic limitations of peptides when used in biological assays [2]. Within the CB1 sequence, LTTFGVHWVRQS, alanine scanning revealed that Phe, His, Trp, and Arg residues mainly contribute to its binding to CD4 [29].

In the work described in the present paper, we developed novel ligand-binding molecules, the so-called "PIN-bodies," with defined specificity, by using PIN as a scaffold protein for anti-CD4 PDP CB1 selected from the antigen-binding pocket of 13B8.2 mAb. In this initial trial, we describe the design, construction, purification, and biological characterization of these PIN-bodies. We then demonstrated that CB1-inserted PIN-bodies bind CD4 more efficiently than soluble PDP CB1. This increased binding was found to be correlated with an increased ability to block antigen presentation by inhibition of IL-2 secretion and to inhibit the one-way and two-way mixed lymphocyte reactions.

Materials and methods

Construction of PIN-bodies. DNA handling and bacterial transformations were performed according to standard procedures, unless otherwise stated. The nucleotide sequence of PDP CB1 was inserted into the PIN gene by a two-step overlapping PCR [30], using 10 pmol of the appropriate oligonucleotide pair (see description in Fig. 1B), 0.5 U of *Tfl* polymerase (Epicentre, Madison, WI), 2 mM dNTP (Invitrogen, Paisley, UK), 37.5 mM $MgCl_2$ (Epicentre), 1× Tfl buffer, and the pET21b plasmid containing the human PIN gene as the DNA matrix [15]. The PCR procedure included a 5-min denaturation step at 94 °C followed by 25 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and ended by a 10-min elongation step at 72 °C. PCR products were run on a 1% (w/v) agarose gel and purified using the QIAquick gel extraction kit and protocol (Qiagen, Hilden, Germany). After digestion with *Nde*I and *Xho*I restriction enzymes, CB1-inserted PIN genes were ligated into the pET21a plasmid, thereby introducing a C-terminal 6-His tag (Novagen VWR International SAS, Fontenay-sous-Bois, France), using 400 U T4 DNA ligase (Promega, Madison, WI) and associated reaction buffer for 18 h at 16 °C. Ligation products were electroporated into TOP10 competent *E. coli* cells (Invitrogen), and the transformed bacteria were plated on LB plates supplemented with 50 μg/ml of ampicillin (LB-amp) and cultured for 18 h at 37 °C. The resulting clones were further screened by PCR amplification of the complete fusion gene as described above. Positive clones, bearing the CB1 sequence inserted between nucleotides coding for PIN residues N33 and I34, R60 and N61, or G79 and Q80, were sequence controlled by the dideoxynucleotide termination sequencing method. For control constructions, a peptide corresponding to the CDR2-L of 13B8.2 variable light chain (LVHDAKTLAEGV) and showing no CD4 reactivity [2] was inserted between the same positions.

Escherichia coli expression of PIN-bodies. BL21(DE3) *E. coli* cells (Invitrogen) were transformed by heat shock with the plasmid preparation from each selected clone and plate-cultured on LB-amp plates for 18 h at 37 °C. Colonies were scrapped by flooding the plates with LB medium and further cultured in 1 L of LB-amp medium at 37 °C under shaking until an A_{600} of 0.5–0.6 was reached (typically in 2–3 h). Expression of PIN-bodies was induced by adding IPTG (Euromedex, Mundolsheim, France) to a final concentration of 1 mM and performed for 18 h at 30 °C. Then, bacteria cells were harvested by centrifugation at 3000*g* for 15 min and subjected to five freeze-thaw cycles. Bacterial pellets were further suspended in sonicating buffer A [20 mM Na_2HPO_4 (pH 6.5), 0.1% Triton X-100, 1 mM $MgCl_2$, 20 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol] and sonicated twice for 30 s on ice. A protease inhibitor cocktail (Roche, Indianapolis, IN), a 1 mg/ml lysozyme solution, and DNase I were then