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The Dac-tag, an affinity tag based on penicillin-binding protein 5

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ABSTRACT

Penicillin-binding protein 5 (PBP5), a product of the *Escherichia coli* gene *dacA*, possesses some β-lactamase activity. On binding to penicillin or related antibiotics via an ester bond, it deacylates and destroys them functionally by opening the β-lactam ring. This process takes several minutes. We exploited this process and showed that a fragment of PBP5 can be used as a reversible and monomeric affinity tag. At ambient temperature (e.g., 22 °C), a PBP5 fragment binds rapidly and specifically to ampicillin Sepharose. Release can be facilitated either by eluting with 10 mM ampicillin or in a ligand-free manner by incubation in the cold (1–10 °C) in the presence of 5% glycerol. The "Dac-tag", named with reference to the gene *dacA*, allows the isolation of remarkably pure fusion protein from a wide variety of expression systems, including (in particular) eukaryotic expression systems.

For protein purification, ectopically expressed proteins are normally tagged to allow the use of affinity chromatography. Most of the commonly used tags are optimized for bacterial expression. The GST-tag, which exploits the interaction between glutathione *S*-transferase (GST)[1] and glutathione (GSH), is probably the most commonly used affinity tag for protein purification [1,2]. This tag is more powerful in bacteria than in eukaryotic cells because eukaryotic cells contain several isoforms of endogenous GST and also GSH, which interfere with the purification of ectopically expressed GST-tagged proteins. The maltose-binding protein (MBP)-tag provides very good selectivity and is often used to improve solubility of target proteins. The drawback of this tag is its size of just over 40 kDa [3]. In contrast, the short polyhistidine-tag consists of an amino acid repeat of 6 or more histidine residues and is bound to an immobilized metal, such as Ni^{2+}-nitrilotriacetic acid (NTA) Sepharose or TALON resin, and eluted with high concentrations of imidazole [4–7]. Although the polyhistidine-tag works reasonably well for bacterial expression, particularly with highly expressed proteins, its use in eukaryotic cells is restricted to highly expressed proteins due to the relatively low selectivity of the affinity media. A number of proteins contain "natural" His-tags within their amino acid sequence. Metal resins also act as anion exchangers and bind to a variety of contaminants. Furthermore, subsequent removal of the eluent imidazole is imperative for most downstream applications, introducing a second purification step, which may reduce yields. Other popular tags include epitope tags, which are recognized by specific antibodies, such as the FLAG-tag [8], the Myc-tag [9], and the hemagglutinin (HA)-tag [10]. They are short, inert, and suitable for immune detection by Western blotting and for small-scale purification, but not for medium- or large-scale protein purification, because the eluents are peptides or antibodies that are expensive and contaminate the samples. Other proprietor tags, such as the Strep-tag, the S-tag, and the Halo-tag, have been introduced during the past couple of years. For the isolation of very pure native fusion proteins, tandem affinity purification (TAP) methods have been introduced. These methods share a principle by using two or three different tags (e.g., GST, His_6, Ca^{2+}-binding domains, streptavidin, protein G-binding domains, HA, Myc), sometimes separated by a protease cleavage site [11–13]. TAP-tag methods are more complex and more expensive than a one-step procedure. They are also more difficult to scale up, and yield is diminished by the need for at least two chromatographic steps.

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[1] *Abbreviations used:* GST, glutathione *S*-transferase; GSH, glutathione; HA, hemagglutinin; TAP, tandem affinity purification; PBP, penicillin-binding protein; PBP5, penicillin-binding protein 5; NHS, *N*-hydroxysuccinic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; cDNA, complementary DNA; TEV, tobacco etch virus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; LB, Luria–Bertani; SD, synthetic deficient; CHC, clathrin heavy chain; PR, peroxiredoxin-1; fc, final concentration.

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When working with proteins purified from eukaryotic expression systems, we noticed that many of the currently available tags have limitations, especially when we aimed for high protein purity at low expression levels. To overcome this problem, we set out to develop a new tag using a ligand–enzyme pair that is alien to eukaryotic cells in the hope that this approach would yield a tag with great specificity and purity. In particular, we exploited the interaction between a penicillin and an *Escherichia coli* penicillin-binding protein.

A unique feature of bacterial cells is the composition of their cell wall, which, in contrast to plants and fungi, is made up of peptidoglycan chains. Transpeptidases crosslink these chains, creating tetrapeptide bridges made up of L- and D-amino acids. The extent of crosslinking is regulated by carboxypeptidases, which are therapeutic targets of β-lactam antibiotics, encompassing the penicillins and derivatives thereof. The penicillin drug targets are referred to as penicillin-binding proteins (PBPs), and *E. coli* naturally expresses 11 PBPs: PBP1a, PBP1b, PBP1c, PBP2, PBP3, PBP4, PBP4b, PBP5, PBP6, PBP6b, and PBP7 [14]. They also have names that describe their catalytic activity. One of them, PBP5 or D-alanyl-D-alanine carboxypeptidase fraction A, is encoded by the *dacA* gene in *E. coli*. PBP5 (acc. no. P0AEB2) has been very well characterized. Many of its biochemical properties have been elucidated, and the crystal structure has been solved [15–20]. Amino acids 37 to 297 form the catalytic domain. This is followed by a finger-like domain made up of β-sheets and a membrane attachment sequence at the extreme C terminus. The N-terminal 36 amino acids are probably not involved in the catalytic mechanism. PBP5 possesses β-lactamase activity and deacylates penicillin G with a half-life of approximately 9 min [16,17,20,21]. This implies that the binding to penicillin derivatives is reversible, which in turn makes the catalytic domain of PBP5 a candidate for a reversible protein tag. Furthermore, PBP5 exists as a monomer, which has certain advantages for a protein tag, for example, when protein dimers or oligomers need to be purified.

Materials and methods

Consumables

If not stated otherwise, all chemicals were obtained from VWR International and purchased at the highest available quality. GSH Sepharose and *N*-hydroxysuccinic acid (NHS)-activated Sepharose were purchased from GE Healthcare Life Sciences (UK). Ampicillin was obtained from Calbiochem (UK). Prestained protein marker SeeBlue Plus, Cellfectin II, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), gentamycin, and oligonucleotides were obtained from Invitrogen. Insect medium, Insect Express, was obtained from Lonza. The marker used in Fig. 4E was Marker II from Peqlab (Germany). For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the SciePlas 20 × 20-cm system was used. Restriction enzymes were obtained from Fermentas, agarose was from Melford, and protein stain was Instant Blue from Expedeon.

Cloning of mammalian expression constructs

For PBP5-GFP, the *dacA* fragment Met37-Asp392 of acc. no. AP_001281 was cloned from genomic DNA of *E. coli* strain JM109 by polymerase chain reaction (PCR) using the primers ATCCGCTAGCCACCATGATCCCGGGTGTACCGC and GTAAGCTTGGGCCCCTGGAACAGAACTTCCAGATCAATGATTTTGCCGAAGAAGTACC so that a site for PreScission Protease and a multicloning site were added. For the shorter Dac-GFP, the *dacA* fragment Met37-Pro297 was cloned by PCR using ATCCGCTAGCCACCATGATCCCGGGTGTACCGC and GATAAGCTTTGGGTTAACGGTTTCAAAGAAACG. The PCR fragments were cloned into the *Nhe*I and *Hin*dIII sites of the pEGFP-N1 vector. To express Dac-SPAK, the region coding for amino acids 37 to 297 was amplified by PCR and cloned into the pcDNA3.1 vector as a *Hin*dIII-*Not*I fragment upstream of a multicloning site to clone other complementary DNAs (cDNAs) downstream in frame with it. The cloning of SPAK-STE20/SPS1-related proline/alanine-rich kinase (NCBI acc. no. AF099989) has been described previously [22]. The full-length SPAK cDNA has been cloned C-terminally from the Dac-tag using *Bam*HI-*Eco*RV-*Not*I three-way ligation. To express GST-SPAK, the cDNA was cloned into the pcDNA3.1 vector with the same cloning strategy.

Cloning of PBP5 amino acids 37 to 297 into pET24a

pET24a Dac was created by amplifying the Dac insert from the mammalian Dac-GFP vector and subcloning into the *Nde*I and *Not*I sites of pET24a.

Cloning of pFB-Dac-GFP construct for baculovirus

PBP5 residues 37 to 297 with a modified start (MSVPG) were amplified using CGGTCCGAAACCATGTCCGTGCCGGGTGTACCGCAGATCGAT and GCGGATCCTGGGTTAACGGTTTCAAAGAAACGGAAGC to provide an *Rsr*II-*Bam*HI insert. The GFP was amplified using primers GGATCCGTGAGCAAGGGCGAGGAGCTGTTC and GAATTCTTACTTGTACAGCTCGTCCATGCCGA with a 5′ *Bam*HI and 3′ *Eco*RI site (plus stop codon), and then the two Dac and GFP fragments were ligated to the pFB backbone in a three-way *Rsr*II-*Bam*HI-*Eco*RI ligation.

Cloning of pFB-Dac-ATG7 construct for baculovirus

For a pFast-Bac-Dual-Dac-TEV-parent vector, a *Bgl*II-*Eco*RI flanked Dac-tobacco etch virus (TEV) fragment was amplified from a modified Dac clone (N terminus changed from MIPGVP to MSAIGVP using GTACATGTCTGCAATCCCGGGTGTACCGCAC) using GTAGATCTCAACATGTCTGCAATCCCGGGTTACCG and ACGAATTCCGATCGGGATCCGCCCTGAAAATACAGGTTTTCTGGGTTAACG and then subcloned into the *Bam*HI-*Eco*RI sites of pFBDual to give the final vector. cDNA coding for human ATG7 isoform b (NP_001129503.2) was amplified from IMAGE consortium EST 3504204, cloned into vector pSC-B (Stratagene), and fully sequenced. This sequence was then converted to the isoform a sequence (NCBI acc. no. NM_006395.2) by PCR mutagenesis. Following conversion to the isoform a sequence, the insert was subcloned into pFastBac-Dual-Dac-TEV using restriction sites *Bam*HI/*Not*I to generate a construct for expression of Dac-TEV-ATG7 isoform a.

Cloning of pFB dual His_6-TEV-Hrt1/Dac-TEV-Cdc53 plasmid for baculovirus

The coding region for yeast (*Saccharomyces cerevisiae*) *hrt1* (NCBI acc. no. NM_001183387.1) was amplified from yeast genomic DNA, adding an *Xho*I site with His_6 and a TEV cleavage site in the 5′ primer and an *Nhe*I site in the 3′ primer. The full-length PCR product was cloned into pSC-B (Stratagene) and sequenced. The His_6-TEV-Hrt1 was subcloned into pFastBac-Dual (Invitrogen) as an *Xho*I/*Nhe*I insert. The coding region for yeast *cdc53* (NCBI acc. no. NM_001180191.1) was also amplified from yeast genomic DNA, adding *Bam*HI and *Not*I restriction sites in the 5′ and 3′ primers, respectively. The full-length PCR product was then cloned into pSC-b and sequenced. *Cdc53* was then subcloned from this vector into pEBG-Dac, a modified pEBG 2T vector in which the GST-tag has been replaced with a Dac-tag as a *Bam*HI/*Not*I insert. The Dac-Cdc53 expression cassette was amplified from this vector,