Affinity Tag for Protein Purification and Detection Based on the Disulfide-Linked Complex of InaD and NorpA

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Michelle E. Kimple and John Sondek

The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

ABSTRACT

Affinity tags are not only used for the expression and purification of recombinant proteins but also for the detection of protein-protein interactions. Common problems with many affinity tags are excessive length, which may interfere with the structure and function of tagged proteins, and low affinity and/or specificity for primary detection and purification agents. Preliminary results suggest that the C-terminal five residues of the *Drosophila* protein NorpA, based on the short, covalent interaction they make with the N-terminal PDZ domain (PDZ1) of InaD, are useful as a general affinity tag. First, a PDZ1-alkaline phosphatase fusion protein specifically detects both its physiological ligand and a heterologous protein expressing the NorpA C-terminal five residues. The interaction of PDZ1 with a NorpA-tagged protein is reversible by a reducing agent, which allows nitrocellulose membranes to be stripped completely and reused. In addition, a NorpA-tagged protein can specifically bind to immobilized PDZ1 resin, while other cellular proteins are washed through. After washing, the NorpA-tagged protein is eluted by a reducing buffer. The NorpA tag's short length makes it the smallest affinity tag available, and its specific and high-affinity interaction with PDZ1 could yield a powerful system that improves on currently available technology.

INTRODUCTION

Inactivation of after-potential D (InaD) is absolutely required in the *Drosophila* phototransduction pathway, a well-characterized, G protein-coupled, phospholipase C-mediated signaling cascade (14,15,21). InaD is composed of five PDZ domains (17,19,20), which were named for the first three proteins in which this protein-binding domain was characterized: "p" for post-synaptic density 95, "d" for discs-large, and "z" for zonular occludens 1 (5,9,12). PDZ domains generally interact with the C-terminal 3–5 amino acids of their protein targets, including the free carboxylate group (7). The two main classes of PDZ domains, type I and type II, are divided based on the amino-acid sequences of their ligands. Type II PDZ domains bind the sequence Φ -x- Φ -COO⁻, where " Φ " is a hydrophobic residue (4).

The type II N-terminal PDZ domain (PDZ1) of InaD binds the C-terminus of no receptor potential A (NorpA), the relevant phospholipase C- β isozyme in the phototransduction pathway (1,3,17,19–21). The crystal structure of PDZ1 in complex with a peptide corresponding to the NorpA C-terminus shows that only the last five residues of NorpA contact PDZ1 and that a disulfide bond is the major intermolecular interaction (10). The short PDZ1 binding motif of NorpA, coupled with its covalent yet dissociable interaction, led to the hypothesis that the NorpA C-terminal residues (Thr-Glu-Phe-Cys-Ala) could be used as an affinity tag for protein detection and purification by appropriately modified PDZ1. The experiments

described here aim to demonstrate that PDZ1 specifically detects not only its physiological ligand but also a heterologous protein expressing the NorpA C-terminal five residues (Figure 1A). The ability of immobilized PDZ1 to specifically purify a NorpA-tagged protein from cell lysate is also examined (Figure 1B). Because of its short, linear recognition sequence and its specific interaction with PDZ1 that is both covalent and dissociable, the NorpA tag could be an improvement on most currently available affinity tags.

MATERIALS AND METHODS

Cloning and Protein Expression

PDZ1 (residues 11–107 of InaD) and CT_{Dm} (residues 863–1095 of NorpA) were cloned into pProEXTM HTa (Invitrogen, Carlsbad, CA, USA) and expressed in BL21(DE3) *E. coli* as previously described (10).

G α i1 was cloned into pProEX HTb (Invitrogen) and expressed in BL21(DE3) *E. coli* as previously described (11). To express G α i1 with the NorpA tail, the G α i1 stop codon was removed using the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), yielding pPro/G α i1-Stopless. Oligonucleotides that encoded for the C-terminal five amino acids of NorpA flanked by *Not*I half-sites (underlined) and *Xba*I half-sites (bolded) were annealed by denaturation at 94°C for 5 min, followed by cooling to room temperature for 15 min [5'-GGCCGCAC-GGAATTTTGTGCCTAAT-3' (sense) and 5'-CTAGATTAGGCACAAAATT-

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CCGTGC-3' (antisense)]. Annealed oligonucleotides were ligated into pPro/G α i1-Stopless cut with *NotI* and *XbaI*. G α i1-NorpA was expressed as described for G α i1.

To create the PDZ1-alkaline phosphatase expression vector, cDNA corresponding to residues 13–107 of InaD was amplified from a plasmid template generously provided by Dr. Bih-hwa Sheih (Vanderbilt University, Nashville, TN, USA) using PDZ1-specific primers. A *KpnI* restriction site (underlined) was inserted into the sense primer, and a *BglIII* restriction site (bolded) was inserted into the antisense primer. The sequences of the PDZ1 primers used were 5'-TGGTACCGAGCTCATTCACATG-TGACCT-3' (sense) and 5'-**CAGATCTTCTTGTCGAAGGTCTGAATC**-TC-3' (antisense). The PDZ1 cDNA was ligated into the pCR[®]2.1-TOPO[®] vector and transformed into TOP10 chemically competent cells using the TOPO TA Cloning[®] System (Invitrogen). The pCR2.1/PDZ1 was purified from an overnight culture of a positive transfor-

mant and digested with *KpnI* and *BglIII*. The purified insert was ligated into pQUANTagen(kx) (PhoA* Color[™] System; Qbiogene, Montreal, QC, Canada), producing pQUANT/PDZ1. PDZ1-alkaline phosphatase was expressed by transforming pQUANT/PDZ1 into XL1-Blue[®] *E. coli* (Stratagene) and inducing it with 0.5 mM IPTG, according to the manufacturer's protocol.

Surface Plasmon Resonance

Biotinylated peptides that corresponded to the C-terminal 12 residues of NorpA (UNC Microprotein Sequencing and Peptide Synthesis Facility, Chapel Hill, NC, USA) were loaded onto a streptavidin-coated surface plasmon resonance chip (BIAcore, Piscataway, NJ, USA). Increasing concentrations of purified PDZ1 were injected over the peptide surfaces at a flow rate of 20 μ L/min for 75 s. Between successive measurements, the surfaces were regenerated with DTT-containing regeneration buffer at 20 μ L/min. Background-corrected sensorgrams were fit to a single-site binding model using the numerical integration function of BIAevaluation[®] 3.2 software (BIAcore AB, Uppsala, Sweden). The observed rate constant (k_{obs} s⁻¹) was derived from a least-squares minimization of the fit to the association phase of the sensorgrams to a 1:1 Langmuir binding equation. The association rate constant, k_{on} (M⁻¹ s⁻¹), was determined as the slope of the line k_{obs} versus PDZ1.

PDZ1-Alkaline Phosphatase Blot Analysis

E. coli samples expressing G α i1 or G α i1-NorpA before and after induction were lysed in denaturing sample buffer and resolved by 15% SDS-PAGE. The samples were standardized using the Bio-Rad[®] Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA)

to ensure the equal protein loading of in each lane (33.9 μ g). (In the case of CT_{Dm}, purified CT_{Dm} was added to uninduced *E. coli* lysates, which were subjected to SDS/PAGE as described earlier.) Separated proteins were transferred to nitrocellulose membranes using a tank transfer system (Trans-Blot[®] Cell; Bio-Rad Laboratories).

Nitrocellulose membranes were blocked, washed, and incubated with 1 mL purified PDZ1-AP in 10 mL 5% milk/TBST for 4 h at room temperature. The membranes were washed again and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, St. Louis, MO, USA) for 10 min. After BCIP/NBT development, some membranes were stained for total protein with a solution of 0.1% (w/v) Amido Black 10B[®] (Bio-Rad Laboratories).

Western Blot Analysis

To test for equivalent G α i1 and G α i1-NorpA levels, *E. coli* samples that expressed G α i1 or G α i1-NorpA before and after induction were subjected to SDS-PAGE and transferred to nitrocellulose as described earlier for PDZ1-alkaline phosphatase blot analysis. The membrane was blocked and immunoblotted with a rabbit anti-G α i1 antibody that recognizes the specific G α i1 sequence CLDRIAQPNYI (Bioscience International, Saco, ME, USA) at a 1:1000 dilution in 5% milk/TBST. The membrane was washed and immunoblotted with a 1:2000 dilution of anti-rabbit-HRP antibody (Amersham Biosciences, Piscataway, NJ, USA). After a final wash, the membrane was developed with ECL[™] western blotting reagents (Amersham Biosciences).

To show that PDZ1 blots can be stripped and reused, *E. coli* samples expressing G α i1-NorpA, with 2.5 μ g purified GST added as a control, were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked and incubated with His₆-PDZ1 for 4 h, washed, and incubated with a 1:1000 dilution of a rabbit anti-His₆ antibody (Covance, Princeton, NJ, USA). The membrane was developed with the ECL system. Immediately following development, the membrane was washed with 10 mM

Figure 1. Schematic detailing protein purification and detection uses for the NorpA tag. (A) A protein labeled with the NorpA C-terminal residues (denoted by *) can be detected from a pool of other proteins by incubation with a PDZ1-alkaline phosphatase fusion, followed by washing and the addition of an alkaline phosphatase substrate (BCIP/NBT). (B) A NorpA-tagged protein (*) can be purified from a pool of other proteins by incubating lysate with immobilized PDZ1. Non-tagged proteins are eluted in the wash step, and the NorpA-tagged protein are eluted with the addition of a reducing agent to break the disulfide bond.

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DTT in TBS and a final wash in TBS alone. The membrane was blocked and incubated with a 1:1000 dilution of a mouse anti-GST antibody (Sigma). The incubation with an anti-mouse-HRP antibody (Amersham Biosciences) allowed for development with ECL.

G α i1 Affinity Purification

Purified His₆-PDZ1 (8 mg) was coupled with 5 mL NHS-activated Sepharose® (Amersham Biosciences) for 3 h at room temperature. The Sepharose was deactivated by incubation in 0.5 M ethanolamine for 3 h, followed by alternating washes in 25 mM Tris-HCl, pH 8.0, and 50 mM sodium acetate, pH 3.0. The beads were equilibrated in binding buffer [10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 500 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet™ P-40, 0.5% (w/v) sodium deoxycholate, 10 mM oxidized glutathione].

Soluble lysate from a 100-mL *E. coli* culture expressing G α i1 or G α i1-NorpA was incubated with 1 mL nickel agarose or 1 mL PDZ1-coupled sepharose, respectively, for 30 min at room temperature. The beads were washed twice with 20 column volumes nickel buffer A (20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 10 mM imidazole, 10% glycerol) or PDZ1 binding buffer. G α i1 or G α i1-NorpA was eluted with nickel buffer B (buffer A + imidazole) or PDZ1 elution buffer (binding buffer + DTT). Samples from each purification step were separated by SDS-PAGE. Gels were stained with Silver Stain Plus® (Bio-Rad Laboratories).

RESULTS

Wild-Type NorpA Sequence Is Optimized for PDZ1 Binding

To demonstrate that PDZ1 specifi-

cally interacts with the wild-type NorpA sequence, surface plasmon resonance studies were conducted in which N-terminally biotinylated NorpA peptides were immobilized on a streptavidin-coated surface plasmon resonance chip, followed by injections of various concentrations of purified PDZ1. The results indicate that only cysteine-containing peptides, both wild-type (Figure 2A; EFCA) and mutant (Figure 2B; EACA) elicit a concentration-dependent binding response to PDZ1. Because the dissociation rate constant (k_{off}) of a covalent interaction is zero, the affinity of the interaction, which is usually communicated as the equilibrium dissociation constant K_D ($k_{\text{off}}/k_{\text{on}}$), is zero, regardless of the value of the association rate constant (k_{on}). Therefore, k_{on} (M⁻¹s⁻¹) is the only meaningful value for the comparison of PDZ1 binding to NorpA peptides. When k_{on} is calculated for the cysteine-containing pep-

Table 1. Calculated Affinities of PDZ1 for Wild-Type and Mutant NorpA Peptides

C-Terminal Sequence	k_{on} ($M^{-1}s^{-1}$)
EFCA	563.5
EACA	1.35
EFSA	none
EFAA	none

k_{on} ($M^{-1}s^{-1}$) was determined by a least-squares linear fit of k_{obs} (s^{-1}) versus the PDZ1 concentration (M). EFSA and EFAA demonstrate no binding to PDZ1; therefore, k_{on} is not calculated (indicated by "none").

Figure 2. PDZ1 binds with high affinity to wild-type NorpA peptide but not to mutants. (A) A biotinylated peptide that corresponds to the C-terminal 12 residues of NorpA (EFCA) was immobilized on a streptavidin-coated surface plasmon resonance chip and various concentrations of PDZ1 flowed over. The wild-type NorpA peptide shows a strong concentration-dependent binding response to PDZ1. (B) A peptide that corresponded to a Phe-to-Ala mutation (EACA) exhibits a weak concentration-dependent binding response to PDZ1. (C) PDZ1 did not exhibit any binding to a peptide that corresponded to an isosteric Cys-to-Ser mutation (EFSA). The sensorgram was nearly identical to panel D, where the immobilized peptide corresponded to a Cys-to-Ala mutation (EFAA).

tides EFCA and EACA, a 400-fold decrease in affinity is observed for the mutation of Phe(-2) to Ala ($k_{on} = 563.5 M^{-1}s^{-1}$ and $1.35 M^{-1}s^{-1}$, respectively) (Table 1). The disulfide bond is clearly the most important interaction, though, since the mutation of Cys(-1) to Ser or Ala completely abolishes binding (Figure 3, C and D, EFSA and EFAA). In addition, cysteine hydrogen-bonding plays no part in the NorpA/PDZ1 interaction because the isosteric Cys-to-Ser mutation had the same effect as the nonconservative Cys-to-Ala mutation. These results indicate that the wild-type NorpA sequence is optimized for PDZ1 binding, which is important because a motif search of the Swiss-Prot database (<http://motif.genome.ad.jp/>) reveals that only five other proteins have the same three C-terminal residues as NorpA (F-C-A-COO⁻). Even a more general Φ -C- Φ -COO⁻ sequence only occurs in 200 different proteins in the Swiss-Prot database.

PDZ1-Alkaline Phosphatase Binds to CT_{Dm} Specifically

To show that PDZ1-alkaline phosphatase can be used to specifically detect its physiological ligand from a pool of other proteins, varying amounts of purified CT_{Dm} were added to BL21 (DE3) whole-cell lysate (33.9 μ g lysate protein) and separated by PAGE, along with a control lane of purified CT_{Dm} without lysate. Protein bands were transferred to a nitrocellulose membrane, blocked, and incubated with PDZ1-alkaline phosphatase for 4 h. After we had washed the membrane

extensively, it was developed with BCIP/NBT. Fifty nanograms of CT_{Dm} are faintly detected out of 33.9 μ g total cellular protein (CT_{Dm} less than 1/600th total protein), while 100 ng are clearly detected by PDZ1-alkaline phosphatase (Figure 3A). Since CT_{Dm} alone does not react with BCIP/NBT (data not shown), any color change is a result of bound PDZ1-alkaline phosphatase. Additionally, because no other bands react with BCIP/NBT, PDZ1-alkaline phosphatase interacts specifically with CT_{Dm}, even in a background of cellular lysate (Figure 3B).

Addition of the NorpA Tail Confers PDZ1 Binding to Heterologous Proteins

The C-terminal five residues of NorpA were spliced onto G α i1, a signaling protein that does not normally interact with PDZ1, to demonstrate that the addition of these residues is sufficient to confer PDZ1-binding ability. Bacterial samples that expressed either G α i1 or G α i1-NorpA were separated by gel electrophoresis and transferred to a nitrocellulose membrane, along with purified CT_{Dm} as a positive control. As previously described for CT_{Dm} (Figure 3), the membrane was blocked, incubated with PDZ1-alkaline phosphatase, washed, and developed with BCIP/NBT. PDZ1-alkaline phosphatase specifically detects G α i1-NorpA but not untagged G α i1 (Figure 4A) even in a background of cellular lysate (Figure 4B). As a control for G α i1 expression, a duplicate blot was probed with a rabbit anti-G α i1 primary antibody, followed by an anti-rabbit HRP-conjugated secondary antibody. A chemiluminescent assay clearly shows that G α i1 and G α i1-NorpA were both similarly expressed at high levels (Figure 4C).

PDZ1 Bound to a NorpA-Tagged Protein Is Easily Dissociable

There are several protocols for stripping antibodies from membranes to re-probe, but most involve high temperature (70°C), detergents (SDS), high concentrations of denaturants [200 mM β -mercaptoethanol (β -ME)], and may not be reproducible. To demonstrate further the usefulness of the NorpA tag as a protein detection tool, G α i1-expressing

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E. coli cell lysates spiked with 2.5 μ g purified GST were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with His₆-PDZ1. The PDZ1/G α i1-NorpA interaction was detected by immunoblotting with a rabbit anti-His₆ primary antibody, followed by an anti-rabbit-HRP secondary antibody (Figure 5, left panel). PDZ1 was then stripped from the membrane by the addition of 10 mM DTT to the TBS wash step. The membrane was incubated with a mouse anti-GST primary antibody, followed by an anti-mouse-HRP secondary antibody. After chemiluminescent analysis, there was no residual signal from the anti-rabbit-HRP antibody (Figure 5, middle panel), which indicated that the entire PDZ1/anti-His₆ antibody/anti-rabbit-HRP antibody complex was stripped from the membrane [along with the nonspecific bands typical of anti-His₆ antibody, which is quite promiscuous (6)]. In addition, the mild

stripping conditions leave the membrane amenable to re-probing with anti-GST antibody. Amido Black staining shows the total protein content of the membrane after both immunoblotting experiments (Figure 5, right panel).

Immobilized PDZ1 Can Purify G α i1-NorpA from Cell Lysate

To demonstrate that the NorpA tag is useful for protein purification, we immobilized PDZ1 on NHS-activated sepharose and incubated the beads with the soluble fraction of the *E. coli* cell lysate expressing G α i1-NorpA. The beads were washed extensively, and the bound G α i1-NorpA was eluted with various concentrations of DTT. Samples of subsequent G α i1-NorpA purification steps from control beads and PDZ1-coupled beads were resolved by SDS-PAGE and silver-stained for total protein. G α i1-NorpA shows very little nonspe-

cific binding to blank beads (data not shown). Coupling PDZ1 with the beads allows for the binding and elution of G α i1-NorpA with increasing concentrations of DTT, with most of the bound G α i1-NorpA eluted with 50 mM DTT. (Figure 6A). The identity of the predominant band in the elution fractions as G α i1 was confirmed by immunoblotting with an anti-G α i1 antibody (data not shown). The G α i1-NorpA in the elution fractions is approximately 85% purified compared to the initial sample. This is compared to His₆-G α i1 purified by conventional Ni²⁺ affinity chromatography (Figure 6B), where the overall yield of purified protein in the elution fractions is greater but much less pure.

DISCUSSION

The optimization of the NorpA tag and PDZ1 detection system should

Figure 3. PDZ1-alkaline phosphatase specifically detects CT_{Dm} in a background of bacterial proteins. (A) Varying amounts of purified CT_{Dm} were added to BL21(DE3) samples and separated by SDS-PAGE, followed by transfer to nitrocellulose membranes. The membrane was blocked and incubated with PDZ1-alkaline phosphatase for 4 h, followed by extensive washing. The color change of the CT_{Dm} band after the addition of BCIP/NBT indicates that PDZ1-alkaline phosphatase interacts specifically with CT_{Dm} in a background of bacterial proteins. (B) The same membrane as in panel A was stained for total protein with an Amido Black solution.

Figure 4. PDZ1-alkaline phosphatase specifically detects Gαi1-NorpA in a background of bacterial proteins. (A) *E. coli* cells expressing Gαi1 or Gαi1-NorpA before and after induction were lysed in SDS sample buffer, and the proteins were separated by denaturing PAGE, followed by transfer to nitrocellulose membranes. Purified CT_{Dm} was used as a positive control. The membrane was incubated with PDZ1-alkaline phosphatase for 4 h, followed by extensive washing. The color change of the Gαi1-NorpA band after the addition of BCIP/NBT indicates that PDZ1-alkaline phosphatase specifically interacts with Gαi1-NorpA in a background of bacterial proteins but not with Gαi1 alone. (B) Identical membrane as in panel A, stained with Amido Black to show the total protein species. (C) Western blot analysis of a duplicate nitrocellulose membrane to show equivalent amounts of Gαi1 and Gαi1-NorpA. Lanes are identical to those in panel A. The membrane was incubated with rabbit anti-Gαi1 with an internal recognition sequence, followed by incubation with α-rabbit-HRP to detect Gαi1 by chemiluminescence.

Figure 5. Membranes incubated with PDZ1 can be easily stripped and reused. A Gαi1-NorpA-expressing *E. coli* sample was spiked with 2.5 µg GST, lysed in SDS sample buffer, and the proteins were separated by SDS-PAGE, followed by transfer to nitrocellulose membranes. The membrane was incubated with His₆-PDZ1 for 4 h, washed, and immunoblotted with anti-His₆ to detect Gαi1-NorpA through its interaction with His₆-PDZ1 (left panel). The complete removal of the His₆-PDZ1/rabbit anti-His₆ antibody/anti-rabbit-HRP antibody complex was attained by washing the membrane in a reducing buffer, as evidenced by subsequent immunoblotting with anti-GST (center panel). After all immunoblots were completed, the membrane was stained for total protein with Amido Black (right panel).

Figure 6. Immobilized PDZ1 can purify Gαi1-NorpA from bacterial lysate. (A) PDZ1-coupled beads can bind Gαi1-NorpA, which is specifically eluted by DTT, as shown by silver staining. (B) As shown by Coomassie® stain, His₆-Gαi1 that was purified by Ni²⁺ agarose was not as enriched in the elution fractions as when it was purified by PDZ1 affinity chromatography.

Table 2. Comparison of NorpA Tag with Commonly Used Affinity and Epitope Tags

Affinity Tag	MW (kDa)	Use		Ligand	Elution	Other Advantages	Other Disadvantages	Other Uses
		Detection	Purification					
NorpA	0.6	yes	yes ^b	Immobilized PDZ1	β-ME, DTT, or glutathione	Very short tag; linear recognition; PDZ1 binds specifically and covalently; no need for antibody	Tag at C-terminus only; elution with reductant may disrupt disulfide-containing proteins	Protein–protein interactions ^c , esp. SPR
c-Myc	1.2	yes	yes ^b	Immobilized anti-c-Myc	High pH	Tag at N- or C-term; linear recognition	anti-c-Myc promiscuous; Low pH elution may cause aggregation	Protein–protein interactions, esp. IP/IB
Hemagglutinin (HA)	3.5	yes	yes ^b	Immobilized anti-HA	Free HA peptide	N- or C-term; linear recognition; anti-HA specific		Protein–protein interactions, esp. IP/IB, ELISA
FLAG [®]	4.0	yes	yes ^b	Immobilized anti-FLAG (M1 or M2)	EDTA (M1), Glycine, or free FLAG	anti-FLAG specific; linear recognition	M1 can only bind FLAG at N-terminus of fusion	Protein–protein interactions, esp. IP/IB, ELISA
GFP	31.0	yes	no	N/A	N/A	Tag at N- or C-term; anti-GFP specific; GFP is fluorescent	Very large tag; some GFP fusions non-specifically targeted to nucleus	Protein–protein interactions, esp. immuno-colocalization
GST	28.0	yes	yes	Immobilized glutathione	Reduced glutathione	Tag at N- or C-term; anti-GST specific	Very large tag; GST dimerizes and is highly antigenic; glutathione may affect disulfide-containing proteins	Protein–protein interactions, esp. pull-down assays, IP/IB
His ₆	0.8–3.0 ^a	yes	yes	Immobilized Ni ²⁺	Low pH, imidazole, or EDTA	Tag at N- or C-terminus; linear recognition; native or denatured protein purification	Nonspecific binding to Ni ²⁺ ; anti-His ₆ promiscuous; pH or imidazole may cause aggregation	Protein–protein interactions, esp. pull-down assays

^aLength depends on cloning vector
^bNon- or semi-reusable affinity resins
^cExamples of protein–protein interaction assays include but are not limited to immunoprecipitation/immunoblotting (IP/IB), affinity pull-down assays, ELISAs, cell immuno-colocalization, and surface plasmon resonance (SPR).

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yield a powerful tool for protein purification and detection that improves on currently available methods. Table 2 shows a summary of several characteristics of the NorpA tag, as compared with commonly used affinity and epitope tags (2,6,8,13,16,18).

Two affinity tags are used regularly for both protein detection and purification, GST and His₆ (2,16,18). Both GST and His₆ tags are quite useful for protein-protein interaction assays such as immunoprecipitation/immunoblotting, ELISAs, cell immuno-colocalization, and surface plasmon resonance; however, both have disadvantages. The 28-kDa GST tag is sometimes larger than the protein or peptide of interest, and it readily homodimerizes, which can lead to experimental artifacts. Conversely, His₆ tags can be as short as six residues, depending on the expression vector, but anti-His₆ antibodies are notoriously promiscuous and may require additional residues for specificity.

In comparison, the five-residue NorpA tag is shorter than any of the commonly used affinity tags and is useful for both protein purification and detection. PDZ1 binds specifically and covalently to the NorpA C-terminus (Figures 2–4) and is dissociable with the addition of a reducing agent such as DTT (Figure 5). In addition, the development of a PDZ1-alkaline phosphatase fusion that can detect NorpA-tagged proteins negates the necessity of primary antibody generation, although an HRP-PDZ1 fusion would be useful for sensitive chemiluminescent detection of NorpA-tagged protein. The NorpA tag can also be used to purify proteins via immobilized PDZ1 (Figure 6). Optimization of the purification protocols, such as the addition of a flexible linker to PDZ1 and different coupling chemistries, could yield a moderately reusable, high-affinity resin comparable to glutathione sepharose. This would make the NorpA C-terminus one of the most useful affinity tags, comparable with the His₆ tag in length but potentially more useful for protein detection because of the specific, high-affinity interaction of NorpA with PDZ1. Specifically, the covalent interaction between PDZ1 and NorpA is ideal for protein-protein interaction techniques such as surface plasmon resonance, where a strong attachment of the

protein of interest to the biosensor chip is required, but the covalent attachment of proteins via primary amino groups can sometimes result in the loss of biological activity. A PDZ1 surface would “catch” NorpA-tagged proteins, which would then be analyzed for their binding to various ligands. The entire surface could be regenerated by flowing over a reducing buffer, which would allow the covalent attachment of a different NorpA-tagged protein for a new set of binding experiments.

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Address correspondence to:

Dr. John Sondek
UNC-CH Department of Pharmacology,
CB# 7365
Chapel Hill, NC 27599-7365, USA
e-mail: sondek@med.unc.edu