A rapid and universal tandem-purification strategy for recombinant proteins

ANDREW J. MCCLUSKEY,[1,2,4] GREGORY M.K. POON,[2,4] AND JEAN GARIÉPY[1,2,3]

[1]Department of Pharmaceutical Sciences, University of Toronto, Ontario M5S 3M2, Canada
[2]Ontario Cancer Institute, University Health Network, Ontario M5G 2M9, Canada
[3]Department of Medical Biophysics, University of Toronto, Ontario M5G 2M9, Canada

(RECEIVED March 25, 2007; FINAL REVISION September 5, 2007; ACCEPTED September 16, 2007)

Abstract

A major goal in the production of therapeutic proteins, subunit vaccines, as well as recombinant proteins needed for structure determination and structural proteomics is their recovery in a pure and functional state using the simplest purification procedures. Here, we report the design and use of a novel tandem $(His)_6$-calmodulin (HiCaM) fusion tag that combines two distinct purification strategies, namely, immobilized metal affinity (IMAC) and hydrophobic interaction chromatography (HIC), in a simple two-step procedure. Two model constructs were generated by fusing the HiCaM purification tag to the N terminus of either the enhanced green fluorescent protein (eGFP) or the human tumor suppressor protein p53. These fusion constructs were abundantly expressed in *Escherichia coli* and rapidly purified from cleared lysates by tandem IMAC/HIC to near homogeneity under native conditions. Cleavage at a thrombin recognition site between the HiCaM-tag and the constructs readily produced untagged, functional versions of eGFP and human p53 that were >97% pure. The HiCaM purification strategy is rapid, makes use of widely available, high-capacity, and inexpensive matrices, and therefore represents an excellent approach for large-scale purification of recombinant proteins as well as small-scale protein array designs.

Keywords: protein purification; immobilized metal-affinity chromatography; hydrophobic interaction chromatography; tandem-affinity purification

Supplemental material: see www.proteinscience.org

A quarter of all new pharmaceuticals entering the marketplace are recombinant therapeutic proteins (Pavlou and Reichert 2004; Walsh 2005). A recurrent challenge in manufacturing such proteins has been the development of rapid, robust, and economical protein-purification methods. A similar requirement for homogeneous preparations exists for proteins intended for structural solution by X-ray crystallography and NMR spectroscopy. Finally, proteomic approaches linked to mass spectrometry require a universal purification approach for the recovery of distinct, low-abundance proteins from complex yeast and mammalian cell lysates (Rigaut et al. 1999; Puig et al. 2001). The purification of recombinant proteins has been simplified by affinity chromatographic strategies, in which a peptide or protein-affinity tag, cloned in-frame with the target construct, selectively interacts with a ligand that has been immobilized on a solid support. The most common affinity tag is an oligohistidine tag, typically $(His)_6$, which selectively binds to transition

[4]These authors contributed equally to this work.

Reprint requests to: Jean Gariépy, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario M5G 2M9, Canada; e-mail: gariepy@uhnres.utoronto.ca; fax: (416) 946-6529.

Abbreviations: IMAC, immobilized metal-affinity chromatography; HIC, hydrophobic interaction chromatography; CaM, calmodulin; CBP, calmodulin-binding peptide; TAP, tandem affinity purification; eGFP, enhanced green fluorescent protein; HiCaM, $(His)_6$-calmodulin; GST, glutathione *S*-transferase; MBP, maltose-binding protein; ProtA, protein A.

Article published online ahead of print. Article and publication date are at http://www.proteinscience.org/cgi/doi/10.1110/ps.072894407.

metal ions such as Ni^{2+} and Co^{2+} in a procedure known as immobilized metal-affinity chromatography (IMAC). Although IMAC is routinely used for the purification of recombinant proteins, purity under native conditions is often suboptimal due to nonspecific binding and coelution of host (particularly eukaryotic) proteins with His-tagged targets (Lichty et al. 2005).

In addition to IMAC, other purification tags have been developed including natural or designed peptides as well as protein modules such as maltose-binding protein (MBP), protein A (ProtA), and glutathione *S*-transferase (GST) (Stevens 2000). As in the case of IMAC, none of these purification tags alone is sufficient in most instances to purify recombinant proteins to homogeneity, starting from bacterial or eukaryotic cell extracts. This challenge has led to an array of dual (and multiple) affinity tags in order to improve protein expression (Sachdev and Chirgwin 1998) or product purity (Lichty et al. 2005). These complex tags typically combine the $(His)_6$ peptide with other affinity tags or epitopes such as calmodulin binding peptide (CBP) (Honey et al. 2001), GST (Panagiotidis and Silverstein 1995; Coulombe and Meloche 2002), MBP (Kapust and Waugh 1999; Donnelly et al. 2006; Tropea et al. 2007), thioredoxin (Yeliseev et al. 2007), Strep tags (Mueller et al. 2003; Yeliseev et al. 2007), hemagglutinin (Winkler et al. 1998; Honey et al. 2001), the HIV-Tat peptide (Goda et al. 2007), and the FLAG epitope (Pathak and Imperiali 1997). These approaches vary significantly in terms of product purity, yield, purification conditions, procedural complexity, and cost. An ideal multiple affinity purification procedure should exhibit the following features: (1) substantial gain in purity, (2) small tag, (3) complete removal of the affinity tag after purification, (4) rapid and robust procedures (requiring few or no buffer exchanges between steps), (5) compatibility with native conditions, and (6) low cost. We have devised a novel dual-purification tag that addresses these criteria by combining a $(His)_6$ peptide with calmodulin (CaM) and fusing it to the target protein via a thrombin cleavage site. CaM is a small, water-soluble protein that lacks disulfide bridges and folds spontaneously in solution into a well-defined domain-like structure. Importantly, the Ca^{2+}-dependent affinity of CaM for inexpensive hydrophobic matrices such as phenyl sepharose has been the basis for its purification (Gopalakrishna and Anderson 1982) and, more recently, for CaM fusion proteins (Vaillancourt et al. 2000; Melkko and Neri 2003). Our novel $(His)_6$-CaM (HiCaM) dual-purification tag thus couples IMAC and hydrophobic interaction chromatography (HIC). Using the enhanced green fluorescent protein (eGFP) and the human p53 tumor-suppressor protein as examples, we demonstrated that the sequential IMAC and HIC purification of N-terminally HiCaM-tagged proteins affords a rapid, simple, and inexpensive procedure that yields highly pure, untagged preparations of these two proteins.

Results

Expression and purification of HiCaM-tagged eGFP and p53(1-360) by tandem IMAC/HIC

A novel and universal dual-affinity purification tag, HiCaM-tag, was designed to address the need for rapid and effective purification of recombinant proteins to homogeneity under native conditions. Specifically, the HiCaM-tag (Fig. 1) was fused to the N terminus of either eGFP or p53(1-360) via a thrombin cleavage site, and the resulting protein constructs were expressed in *Escherichia coli*. Both fusion proteins were well-expressed to over 10 mg/L culture (Table 1), indicating that the addition of the HiCaM-tag did not adversely affect their expression.

HiCaM-tagged protein samples were then purified from cleared lysates by IMAC on Ni-NTA sepharose and followed immediately by HIC on phenyl sepharose (Fig. 2A). Since the buffers for IMAC and HIC are compatible, buffer exchange is unnecessary and the IMAC eluate can be applied directly onto phenyl sepharose. SDS-PAGE analysis of fractions collected at various steps showed contaminating protein bands in the eluates from IMAC or HIC alone (Fig. 2B, lanes 4 and 7), whereas coupled IMAC/HIC purification profiles appeared homogeneous (Fig. 2B, lanes 10–12). Purified, untagged proteins were

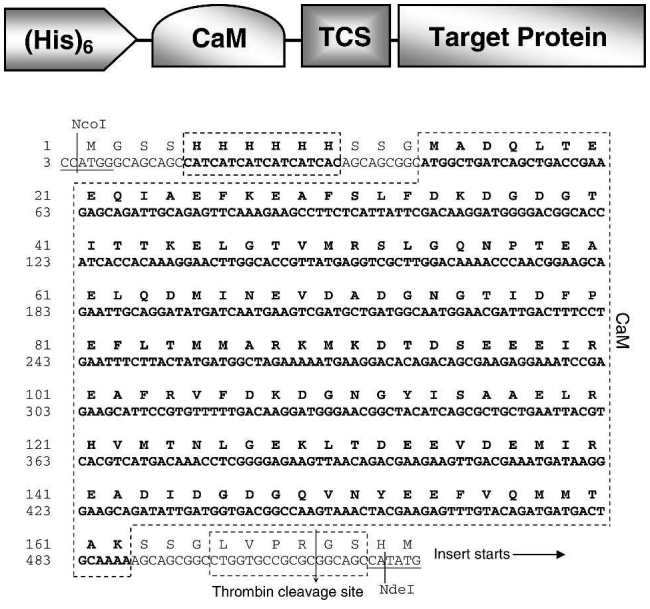

Figure 1. Construction of the HiCaM purification tag. The HiCaM-tag combines a $(His)_6$ sequence for IMAC purification, followed by CaM for HIC purification with phenyl sepharose. The terminal NcoI/NdeI restriction sites (underlined) allow the DNA sequence coding for the tag to be inserted directly into a pET15b vector 5′ to the multiple cloning site. The $(His)_6$-tag and thrombin cleavage sequences are highlighted in blue and red, respectively.

Table 1. *Summary of tandem IMAC/HIC purification of HiCaM-tagged recombinant constructs (per litre culture)*[a]

	eGFP			
	Total protein/mg	Purity/%[b]	Yield/mg[c]	Efficiency/%[d]
Cleared lysate	203 ± 14	23 ± 4	47 ± 9	
IMAC eluate	42 ± 3	69 ± 5	29 ± 3	62 ± 14
HIC eluate	27 ± 3	93 ± 4	25 ± 3	54 ± 12
HIC/on-column cleavage	23 ± 3	98 ± 1	23 ± 3[e]	48 ± 11[e]
	p53(1–360)			
	Total protein/mg	Purity/%[b]	Yield/mg[c]	Efficiency/%[d]
Cleared lysate	220 ± 15	5 ± 3	11 ± 7	
IMAC eluate	5.5 ± 1.0	43 ± 5	2.4 ± 0.5	22 ± 14
HIC eluate	2.5 ± 0.7	92 ± 5	2.3 ± 0.7	21 ± 14
HIC/on-column cleavage	1.3 ± 0.3	98 ± 1	1.3 ± 0.3[e]	12 ± 7[e]

[a] Values are averages of experiments performed in triplicate ± standard error.
[b] Determined by densitometric analysis of Coomassie-stained SDS-PAGE.
[c] Yield = total protein × purity.
[d] Efficiency is calculated as the residual yield at each step relative to the cleared lysate.
[e] Values represent purified, untagged proteins.

recovered, in separate experiments, in an off-column procedure or directly on-column by treatment with thrombin. In the off-column method, tagged proteins were eluted from phenyl sepharose in the presence of EGTA, which was then titrated with excess Ca^{2+} before addition of thrombin. For on-column cleavage, washed resin was resuspended and treated with thrombin to release untagged proteins in the flow-through. Densitometric analyses (Fig. 2C) indicated that products from both methods were essentially identical in purity (>97%), although on-column cleavage represents a simpler approach at a small cost in terms of yield (Table 1). Final recovery of purified eGFP and p53 (1-360) was in excess of 15 (32%) and 1.3 mg (12%) per liter of culture, respectively, based on 2 mL each of Ni-NTA and phenyl sepharose. Since significant amounts of target constructs appeared in the column flow-through (Fig. 2B), it is likely that an appropriate scale-up would further increase the yield.

To verify that the tandem IMAC/HIC purification of HiCaM-tagged constructs under native conditions produced functional proteins, the excitation and emission spectra of the purified, untagged eGFP were recorded and confirmed the recovery of a fluorescent protein (Fig. 3A; Shaner et al. 2005). Also, purified, untagged human p53(1-360) was shown to bind to a recognition DNA sequence by gel mobility shift (Fig. 3B). Finally, SDS-PAGE analysis after glutaraldehyde cross-linking of p53(1-360) revealed a ladder of bands characteristic of tetramers formed in solution via its tetramerization domain (residues 325–355) (Fig. 3C; Lee et al. 1994; Poon et al. 2007). Therefore, the native conformation of both proteins was not compromised by their expression as fusion proteins with an N-terminal HiCaM-tag.

Comparison with another dual-tag purification strategy

Although tandem IMAC/HIC purification of HiCaM-tagged eGFP and p53(1-360) produced essentially homogeneous preparations, we also purified, for comparison, eGFP tagged with an N-terminal ''TAP tag'' (see detailed procedure in the Supplemental material), which consists of two IgG-binding domains of ProtA and CBP, separated by a cleavage site for the TEV protease (Rigaut et al. 1999; Puig et al. 2001). The TAP tag is widely used to purify protein–protein complexes in proteomic analysis. Briefly, the TAP-tagged construct was purified on IgG-sepharose (which interacts with ProtA), released by TEV cleavage, followed by a second purification on CaM-sepharose (which binds CBP in the presence of Ca^{2+}). The procedure was adjusted to use the same volumes of the two resins and their respective washing buffers as the HiCaM procedure described in this report. Under these conditions, TAP-tag purification produced eGFP at a purity and efficiency (fraction of purified target recovered) of (94% ± 2%) and (24% ± 9%) (Supplemental Fig. S1), respectively, compared with (98% ± 1%) and (48% ± 11%) for the HiCaM procedure (Table 1). Thus, for the model protein eGFP, both schemes resulted in similarly pure proteins, but the tandem IMAC/HIC gave a twofold increase in efficiency. In addition, the TAP-tag procedure required a buffer change after TEV cleavage for binding onto CaM-sepharose, and the final purified TAP-tagged product was CBP-eGFP, not untagged eGFP. The presence of this 28 amino acid tag, however, did not qualitatively affect the fluorescent properties of the eGFP protein. In addition, the cost of the TAP-tag purification approach (for the resins and TEV protease) was significantly higher than the HiCaM procedure (Supplemental Table S2).

Discussion

A new method for purifying recombinant proteins was developed by fusing a $(His)_6$-CaM (HiCaM) tag at the N terminus of target proteins (Fig. 1). As examples, we have purified eGFP and p53(1-360) under native conditions to apparent homogeneity (as judged by densitometric analysis of Coomassie-stained SDS–polyacrylamide gels) by a tandem purification strategy that combines IMAC and HIC in a simple procedure (Fig. 2A). The level of purity observed after tandem IMAC/HIC was not reproduced by either IMAC or HIC alone, even after adjustment of wash volumes (Fig. 2B,C). Moreover, the recovered target proteins were functional after removing the HiCaM-tag (native fluoresence

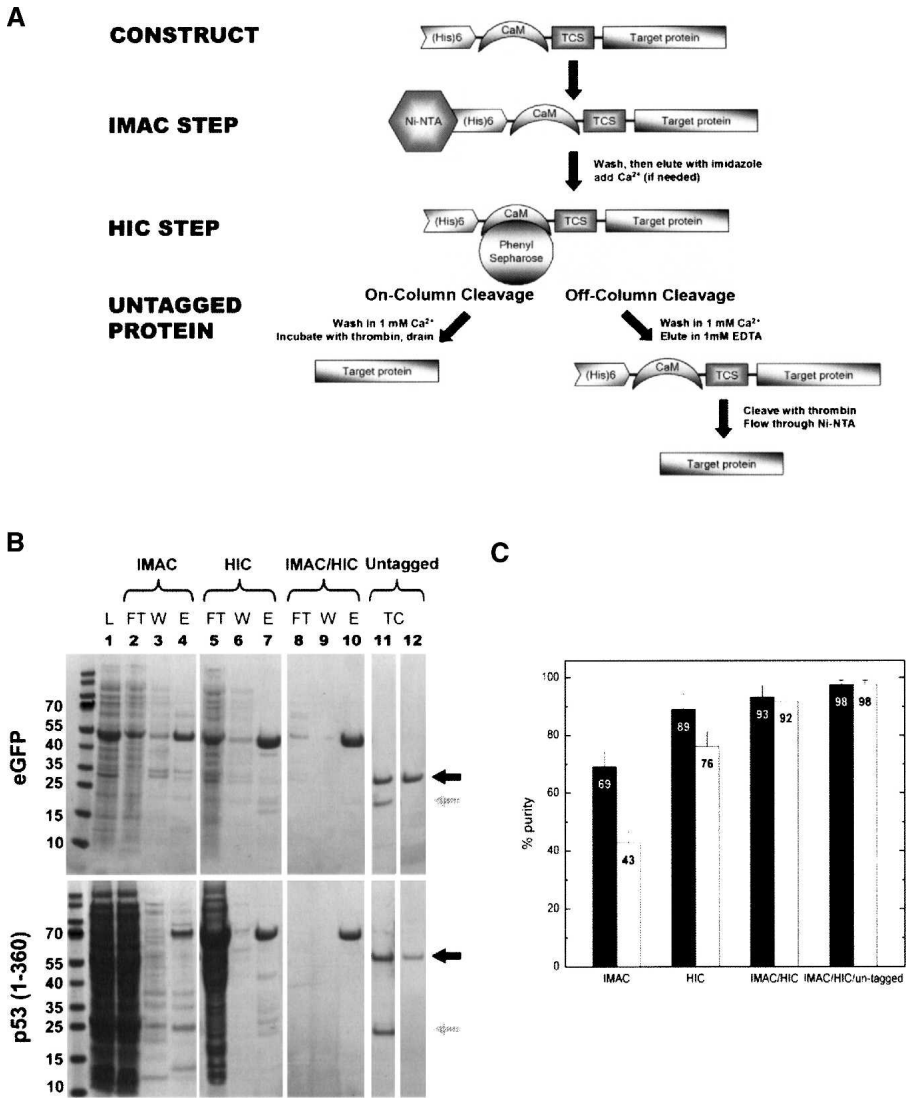

Figure 2. IMAC/HIC purification of HiCaM-tagged proteins. (*A*) The N-terminal HiCaM purification tag combines a $(His)_6$ sequence for IMAC purification, followed by a CaM module for HIC purification with phenyl sepharose. By virtue of the thrombin cleavage site (TCS), the target protein can either be cleaved on-column or eluted and subsequently cleaved with thrombin. See Materials and Methods for detailed purification procedures. (*B*) Aliquots containing 3 μg of total protein corresponding to each purification step were resolved by SDS-PAGE followed by Coomassie Blue staining. HiCaM-tagged proteins were obtained by elution from phenyl sepharose (lanes *7*,*10*) with 50 mM TrisHCl, 1 mM EGTA (pH 7.5), and (for tandem IMAC/HIC purified constructs) subsequently cleaved with thrombin (lane *11*) to release the HiCaM-tag (light arrow) from the target protein (dark arrow). Untagged constructs were also directly obtained by on-column thrombin cleavage (lane *12*). (*C*) Histogram highlighting the purity of proteins (eGFP, solid bars; human p53[1-360], open bars) recovered from IMAC purification step alone, HIC step alone, HiCaM tandem steps, and following the removal of the HiCaM purification tag (derived from densitometry measurements performed on lanes *4*,*7*,*10*,*12* in *B*).

for eGFP; specific DNA binding and tetramerization for p53) (Fig. 3), indicating that the HiCaM-tag, once removed, did not perturb the native conformation of the target proteins.

Features of tandem IMAC/HIC and the $(His)_6$-CaM tag

The contiguous combination of $(His)_6$ and CaM results in a convenient, relatively small (19 kDa) purification tag for tandem IMAC/HIC purification. Though larger than peptide-based affinity tags, the HiCaM-tag is one of the smallest protein-based purification tags known: smaller than MBP (40 kDa), protein G (27 kDa), the popular GST tag (28 kDa), and is only marginally larger than ProtA itself (15 kDa). It makes use of two widely available, high-capacity, and inexpensive matrices (Ni- or Co-NTA and phenyl sepharose). CaM is a highly soluble protein

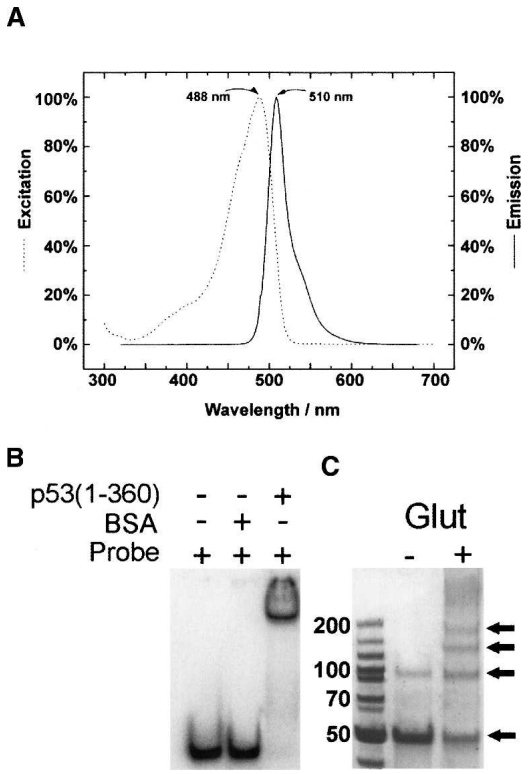

Figure 3. Functional assays of tandem IMAC/HIC-purified constructs following HiCaM-tag removal. (*A*) Fluorescence excitation and emission spectra (excited at 488 nm) of purified eGFP (3 μM) were recorded from 300 to 700 nm at 25°C. (*B*) Human p53(1-360) bound to a specific ^{32}P-labeled DNA recognition sequence (5′-AGGCATGTCTAGGCATGTCT-3′) as monitored by electrophoretic mobility shift (McLure and Lee 1998). (*C*) SDS-PAGE of the p53 construct following chemical cross-linking with glutaraldehyde displays a band-ladder characteristic of tetramer formation via its oligomerization domain (residues 325–355) (Poon et al. 2007).

regardless of its Ca^{2+} load, and did not (as indicated by our soluble yield of tagged eGFP and p53) adversely affect the expression of the two fusion constructs (>10 mg/L culture; Table 1). In fact, a structural homolog of CaM, the calcium-binding protein of *Entamoeba histolytica*, has been used as a solubilizing fusion partner for a recombinant multiple-epitope polypeptide (Reddi et al. 2002).

The use of CaM alone as a fusion tag has been described and reviewed by several researchers (Neri et al. 1995; Schauer-Vukasinovic and Daunert 1999; Vaillancourt et al. 2000; Schauer-Vukasinovic et al. 2002; Melkko and Neri 2003). Since various high-affinity CaM ligands are known (e.g., CBPs, phenothiazines), a number of CaM-specific strategies are, at least in principle, possible in addition to HIC. For example, CaM-tagged GFP has been purified on phenothiazine-silica prepared by Daunert and coworkers (Schauer-Vukasinovic and Daunert 1999; Schauer-Vukasinovic et al. 2002). Unfortunately, to our knowledge, affinity matrices harboring specific ligands for CaM are not available commercially. For practical purposes, therefore, if CaM is to be used as part of a fusion tag, a HIC matrix such as phenyl sepharose is the most appropriate choice, given its robustness, high capacity, wide availability, and very low cost.

Since the buffers for Ni-NTA and phenyl sepharose are compatible, the entire IMAC/HIC procedure can be rapidly performed in a pour-on column format with no delay between purification and minimal optimization of wash and elution conditions for either stage (Fig. 2A). The tight retention and high capacity of the IMAC and HIC matrices for $(His)_6$ and Ca^{2+}-CaM, respectively, permit stringent and extensive washing of the resins. In addition, the disparate nature of the interactions in the IMAC and HIC matrices makes it very unlikely that a host contaminant will have the appropriate physicochemical characteristics for strong interactions with both matrices and sensitivity to imidazole and Ca^{2+}.

Conclusion

Tandem IMAC/HIC purification with the HiCaM-tag provides a novel purification method that offers a rapid and easy way to recover pure proteins at low cost. Two target proteins, in this case eGFP and human p53(1-360), were produced in high yield, suggesting that this process is scalable while maintaining their functional (native) conformations. Although we have engineered the HiCaM-tag in a pET15b vector, it can be easily adapted for cloning into any other expression vector (bacterial, yeast, or mammalian) or baculovirus transfer vector (for expression in insect cells). Overall, the tandem IMAC/HIC strategy allows for the purification of recombinant proteins with little-to-no optimization and advances the field of protein purification in the aspect of achieving greater purity using a simple, two-step procedure.

Materials and Methods

Cloning of constructs

To construct the tandem HiCaM fusion tag (Fig. 1), the gene coding for calmodulin was amplified from a plasmid by PCR using a forward primer that added a NcoI restriction site as well as the $(His)_6$ sequence (underlined) (5′-CCATGGGCAGCAGC CATCATCATCATCATCACAGCAGCGGC-3′) to the 5′-end. A reverse primer appended a thrombin cleavage site (underlined) and NdeI restriction site (5′-AGCAGCGGCCTGGTGCCGC GCGGCAGCCATATG-3′) at the 3′-end. The PCR product was cloned into the NcoI/NdeI sites of a pET15b vector (Novagen) situated upstream of the multiple cloning site, thus replacing the single $(His)_6$-tag of the unmodified vector. Inserts corresponding to eGFP or human p53 (1-360) were then cloned

into the NdeI/BamHI sites of the modified vector by standard procedures. Plasmids were transformed into TOP10 *E. coli* (Invitrogen) and clones were identified by DNA sequencing.

Protein expression and purification

BL21(DE3)Star *E. coli* (Invitrogen) transformants harboring each plasmid were grown in LB broth containing 100 μg/mL ampicillin. Cells were grown in shaking flasks (225 rpm) at 37°C until they reached an OD_{600} of 0.6. Protein expression was induced with 0.75 mM IPTG, and the cultures were maintained overnight with shaking at room temperature. Cell pellets from 1-L cultures (~3 g) were resuspended in 30 mL of Buffer A (50 mM TrisHCl, 1 mM $CaCl_2$, pH 7.5) supplemented with protease inhibitors (Complete Mini EDTA-free; Roche) and a nonspecific nuclease (Benzonase Nuclease, 2.5 kU; Novagen). Cells were lysed by stirring with 10 g of acid-washed glass beads (Sigma) as described elsewhere (Song and Jacques 1997) and cleared by centrifugation at 40,000*g* for 20 min. For tandem IMAC/HIC, the cleared lysate was adjusted to 10 mM imidazole and loaded onto a 2-mL bed of Ni-NTA (Sigma-Aldrich) that had been pre-equilibrated with Buffer A. The loaded resin was washed with 10 mL of Buffer A before eluting the bound protein with 10 mL of Buffer A adjusted to 150 mM imidazole. The IMAC-purified eluate was then directly applied onto a 2 mL bed of phenyl sepharose 6 FF (GE Healthcare) that had been pre-equilibrated with Buffer A and washed with an additional 10 mL of Buffer A. For HIC alone, the wash volume was 20 mL to match the combined wash volumes for tandem IMAC/HIC. Purified protein was recovered by elution with 5 mL of Buffer B (50 mM TrisHCl, 5 mM EGTA, pH 7.5), titrated with 50 μL of 1 M $CaCl_2$, and treated with 2 U/mL thrombin (GE Healthcare). Alternatively, the column was plugged and the resin was resuspended in 5 mL of Buffer B containing 2 U/mL of thrombin. The resin slurry was gently shaken overnight at room temperature. Following thrombin treatment, the untagged protein was drained from the resin and passed through a 0.5 mL bed of *p*-aminobenzamidine agarose (Sigma-Aldrich) to remove residual protease. Protein concentrations at various steps were measured by Coomasie Blue binding (Bio-Rad), using bovine serum albumin as a standard.

Assessment of target purity

Protein samples (3 μg total protein) were analyzed by SDS-PAGE and stained with Coomassie Blue. Gel lanes were scanned by transillumination in a Bio-Rad Gel Doc XR instrument and the purity of the target protein band was quantified by densitometry using the software Quantity One (version 4.6.3, Bio-Rad).

Functional assays of purified, untagged constructs

The excitation and emission spectra (excited at 488 nm) of eGFP were recorded from 300 to 700 nm at 25°C in a Fluoromax-3 Spectrofluorometer. For p53(1-360), a ^{32}P-labeled restriction fragment harboring an icosamer recognition sequence (5′-AGGCATGTCTAGGCATGTCT-3′) (0.10 nM) was incubated alone, with 50 ng/μL BSA or 12 μM p53(1-360) in PBS at 25°C with 2 μg of poly(dI-dC)·poly(dI-dC) for 1 h before separation in a 6% polyacrylamide gel in TBM buffer at 25 V/cm. The gel was then exposed to a phosphor screen for 1 h and imaged in a Typhoon PhosphorImager (GE Healthcare). Human p53(1-360) (7 μM) was also cross-linked with 0.125% glutaraldehyde at 25°C for 12 min. and separated by SDS-PAGE, followed by Coomassie Blue staining (Poon et al. 2007).

Acknowledgments

We are grateful to Drs. Brian Raught, Cheryl H. Arrowsmith, and Mitsu Ikura for the gifts of the TAP tag, human p53, and CaM genes, respectively. This work was supported by grants from the Canadian Breast Cancer Research Alliance in association with the Canadian Cancer Society and from the Canadian Institutes of Health and Research.

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