Protein Interaction Module–assisted Function X (PIMAX) Approach to Producing Challenging Proteins Including Hyperphosphorylated Tau and Active CDK5/p25 Kinase Complex*

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Many biomedically critical proteins are underrepresented in proteomics and biochemical studies because of the difficulty of their production in Escherichia coli. These proteins might possess posttranslational modifications vital to their functions, tend to misfold and be partitioned into bacterial inclusion bodies, or act only in a stoichiometric dimeric complex. Successful production of these proteins requires efficient interaction between these proteins and a specific “facilitator,” such as a protein-modifying enzyme, a molecular chaperone, or a natural physical partner within the dimeric complex. Here we report the design and application of a protein interaction module–assisted function X (PIMAX) system that effectively overcomes these hurdles. By fusing two proteins of interest to a pair of well-studied protein–protein interaction modules, we were able to potentiate the association of these two proteins, resulting in successful production of an enzymatically active cyclin-dependent kinase complex and hyperphosphorylated tau protein, which is intimately linked to Alzheimer disease. Furthermore, using tau isoforms quantitatively phosphorylated by GSK-3β and CDK5 kinases via PIMAX, we demonstrated the hyperphosphorylation-stimulated tau oligomerization in vitro, paving the way for new Alzheimer disease drug discoveries. Vectors for PIMAX can be easily modified to meet the needs of different applications. This approach thus provides a convenient and modular suite with broad implications for proteomics and biomedical research. *Molecular & Cellular Proteomics 14: 10.1074/mcp.O114.044412, 251–262, 2015.

The human genome contains ~30,000 protein-encoding genes, and the proteome is estimated to be at least 10 times larger than the genome (1). A key contributing factor to the proteome’s large size is the number of pervasive posttranslational modifications (PTMs).1 The omnipresence of PTMs affects numerous cellular functions. Certain diseases and cancers have been linked to the deregulation of many PTMs. For example, abnormal hyperphosphorylation of tau and α-synuclein is intimately associated with the onset and progression of Alzheimer disease and Parkinson disease, respectively (2). Germline mutations of the highly related histone acetyltransferases CBP and p300 cause the Rubinstein–Taybi syndromes that predispose patients to tumors, lymphoma, and other diseases (3, 4).

Both enzyme and substrate proteins have been targets for drug development for PTM-related disorders. In the case of Alzheimer disease, for example, the kinases CDK5/p25 and GSK-3β, as well as their disease-related product, hyperphosphorylated tau, are prominent subjects for drug discovery (5). However, protein-modifying enzymes frequently have pleiotropic substrates and functions. Maintaining drug target specificity thus is a great challenge. Approaches to controlling the PTM bearers are often hampered by the lack of a versatile and cost-effective technology for producing recombinant proteins with the desired PTM. For example, of the 25,400-plus human protein structure entries in the RCSB Protein Data Bank as of April 2014, only 647 had the keyword “phosphorylated” (2.5%), and 147 hits (0.64%) had “phosphate” in the structure description. At least 63 of these 147 entries were (co-)structures of synthetic phosphorylated peptides. These numbers fall far behind the estimation (6) that 30% of all human proteins are phosphorylated at any moment in a person’s life, and

1 The abbreviations used are: PTM, posttranslational modification; PIMAX, protein interaction module–assisted function X; GSK-3β, glycogen synthase kinase-3β; CDK, cyclin-dependent kinase; LIC, ligation-independent cloning; Ni-NTA, nickel-nitrilotriacetic acid; PIM, protein interaction module; TEV, ; Cat, catalysis; AD, Alzheimer disease; p-tau, hyperphosphorylated tau; CAF, chaperone-assisted folding; CoP, co-purification; TEV, tobacco etch virus.
thus they reveal the significant lapse in our understanding of the pervasive PTMs. Therefore, a simple method for producing quantitatively modified recombinant proteins will likely benefit many basic and translational research topics.

Misfolding and insolubility are common hurdles in the production of recombinant proteins as well. Many heterologously expressed proteins aggregate and form inclusion bodies, severely limiting the final yield (7). One approach to facilitating the productive folding of recombinant proteins is to overexpress molecular chaperones such as the DnaK/DnaJ and GroEL/GroES systems in the host *Escherichia coli* cells (8). Although a number of proteins have been produced successfully in these sophisticated bacterial strains, cellular growth impairment and unwanted proteolysis after refolding have been reported (9). In contrast to these multicomponent protein folding/disaggregation systems, other proteins exert the molecular chaperone function from within a much smaller ensemble or even without an obligatory cofactor (10). These simpler molecular chaperones include peptidyl prolyl cis-trans isomerase, thioredoxin, and disulfide isomerase dsbA (8, 11–13) and have been fused to target proteins to act as solubility enhancers. The simple operation and minimal strain manipulation make such molecular chaperones particularly attractive in synthesizing recombinant proteins.

Here we present a versatile recombinant protein expression system that overcomes some of the aforementioned challenges. This PIMAX approach takes advantage of Fos and Jun leucine zipper protein–protein interaction modules (PIMs) to assist the association of a protein of interest with its “facilitator.” This facilitator may be an enzyme that yields quantitative modification, a molecular chaperone that improves solubility, or a natural physical partner for the protein of interest, resulting in the assembly of a functional heterodimer. Available PIMAX vectors are easily adaptable for the production of different, otherwise challenging proteins, rendering this system widely applicable and highly versatile.

**EXPERIMENTAL PROCEDURES**

*Enzymes, Chemicals, Media, and Strains—Enzymes used for cloning experiments were purchased from New England Biolabs (Ipswich, MA) unless otherwise stated. Microbiology medium ingredients were bought from Fisher Scientific (Pittsburgh, PA). All chemicals and oligonucleotide primers were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

For bacterial plasmid construction, the bacterial strain DH5α (Invitrogen, Carlsbad, CA) was used. DH5α strains were made chemically competent for transformation using either the CaCl2 method or overnight culture. Cells were induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside at 30 °C for 4 h.

*Plasmids and Recombinant Genes—Key plasmids and their relevant features are summarized in the supplemental material. In addition, their sequences have been uploaded as SerialCloner files. Individual steps for the construction of these plasmids are available from the corresponding author upon request.

All recombinant genes except TcFKBP18 were PCR-amplified from human cDNA or yeast genomic DNA. TcFKBP18 was chemically synthesized by Genscript with codons optimized for *E. coli* expression.
5% glycerol) before elution with 40 ml of elute buffer (250 mM imidazole, 20 mM Tris, pH 8.0, 100 mM NaCl, 5% glycerol). The eluates were concentrated to A280, around 1 by centrifugation through a spin column (Centrifugal Filter Unit, Amicon, Ultra-15, 10K). TEV protease (His-tagged, made in-house), 1:100 (TEV-tau in A280), and final 0.5 mM DTT, 1 mM EDTA were added to the protein and incubated overnight at 34 °C. Protein precipitates were removed by centrifugation at 17,000 × g for 30 min at 4 °C. The supernatant containing tau or p-tau was further enriched via gel filtration chromatography (Superdex 200 column, pressure set to 1.5 bar, flow rate set to 0.4 ml/min). Fractions were examined on SDS-PAGE gels, and those containing tau were pooled and concentrated again by a spin column.

For the examination of their overall phosphorylation status, denatured tau and p-tau were purified via a boiling method that exploited the heat resistance of tau and p-tau (2). In this approach, the conditions for induction and cell lysate preparation were identical to those described above. Before subjecting the crude lysates to heating, we added 500 mM NaCl (final concentration). The solution was then placed in a boiling water bath for 20 min, after which it was chilled on ice for 20 min. Precipitated proteins were removed by centrifugation at 20,800 × g for 30 min at 4 °C. Supernatant containing relatively pure tau and p-tau was collected and stored at −80 °C until use.

FKBP18-CDK5/p25 Purification—Bacterial pellets were suspended in purification buffer (100 mM NaCl, 20 mM Tris, pH 7.4) and lysed first by incubation with 1 mg/ml lysozyme in 1 mM EDTA, 1 mM PMSF and 0.1% Triton X-100 at 30 °C for 1 h and then by sonication (Misonix Sonicator 3000, output level, 4.0; total process time, 5 min; pulse-ON time, 5 s; pulse-OFF time, 5 s). Insoluble particles were removed by centrifugation at 17,000 × g for 40 min at 4 °C, and the supernatant containing the complex of FKB18-CDK5/p25 was bound to Ni-NTA beads after overnight mixing at 4 °C. After the beads had been washed with purification buffer, 200 mM imidazole in purification buffer was used to elute the complex. The eluate was then applied to an anti-FLAG M2 magnetic beads (Sigma) pre-equilibrated with TBS (50 mM Tris, 150 mM NaCl, pH 7.4). The complex was bound to the beads via overnight mixing at room temperature. The slurry was then centrifuged at 6000 × g for 5 min at 4 °C, or via the use of a magnet stand to collect the beads. After being washed with TBS, the beads were suspended in a 5× volume of TBS and subjected to on-bead TEV protease digestion with 1 mM EDTA, 0.5 mM DTT and TEV protease, 1:100 (TEV-tau in A280). After overnight digestion at 4 °C with gentle shaking, the mixture was spun (6000 × g for 5 min at 4 °C) to pellet the beads. The supernatant containing the TEV-liberated complex was collected. Ni-NTA affinity chromatography was further used to remove the His-tagged TEV protease. The unbound materials were concentrated via the use of a spin column. The buffer was changed to storage buffer (20 mM Tris, pH 7.4, 100 mM NaCl) after two rounds of spinning through a spin column. After each round of concentration to 500 μl, the solution was mixed with 8 ml of the storage buffer for another concentration spin.

Tau and p-Tau Aggregation Assay—Detailed procedures of tau and p-tau aggregation reactions are described elsewhere (2). Tau aggregation reactions consisted of 30 μM tau or p-tau, 10 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, with or without 50 μM heparin as the inducer. A 3-ml reaction was assembled and incubated at 37 °C without shaking. At each time point, 10 μl of the reaction material was mixed with SDS-PAGE loading dye and boiled for 3 min before 10% SDS-PAGE and Coomassie Blue R250 staining.

RESULTS

Design of PIMAX and Its Applications—Fig. 1A shows the design concept of PIMAX and its three applications: catalysis for PTMs, chaperone-assisted protein folding, and protein dimer co-purification. The core of these PIMAX applications is a pair of high-affinity and high-specificity protein interaction modules that form a stable heterodimer in cells. When each of the two complementary PIMs is fused to a protein of interest and its facilitator, dimerization of PIMs brings the protein and its facilitator within close range of each other, creating an environment that favors protein–facilitator association. The normal molecular functions of these proteins, such as post-translational modification and molecular chaperone-mediated protein folding, can be facilitated.

To establish the PIMAX system, we first created a series of core plasmids (Fig. 1B). The starting vector was pETDuet-1 (Merck, Darmstadt, Germany) that contained dual T7lac promoters for simultaneous expression of two proteins. Each PIMAX recombinant gene consisted of the PIM, a protease site (thrombin, enterokinase, or TEV; see below), and an LIC box for the insertion of application-specific genes. LIC is a highly effective cloning approach that uses T4 DNA polymerase to generate complementary single-stranded ends following restriction digestion by an eight-base cutter NotI or Asc I (15). Annealing of the complementary strands obviates the need for ligation, thereby expediting the creation of recombinant genes. For protein purification, a hexahistidine (His) tag was included as a part of the Fos leucine zipper fusion protein. This His tag is common in all PIMAX plasmids; a second tag, FLAG, was inserted into the other recombinant gene only for the purpose of co-purification. Additional application-specific modifications are detailed in later sections.

Fig. 1C lists different PIMAX proteins described in this work. Proteins preceding a plus sign were fused to Fos. In addition, vectors containing only one of the two recombinant genes were used as negative controls when needed.

PIMAX-Cat to Produce Hyperphosphorylated Tau—The first case study testing the feasibility of PIMAX was for posttranslational modification (i.e., PIMAX-Cat). We set out to produce hyperphosphorylated tau protein, which is intimately linked to the onset and progression of Alzheimer disease (AD). Tau is a microtubule-associated protein expressed in the central nervous system. Normal tau isolated from normal brains bears two to three phosphate groups per molecule (16, 17). AD tau, in contrast, is hyperphosphorylated, with the number of phosphates per tau increased several-fold. Multiple kinases, including GSK-3β and CDK5, have been shown to phosphorylate tau (18). p-Tau dissociates from microtubules and forms neuronal deposits known as neurofibrillary tangles that are

2 D. Sui, M. Liu, and M.-H. Kuo, unpublished.
hypothesized to be an underlying cause of cognitive impair-
ment and neuronal death in AD (19). However, animal and
tissue culture studies suggest that tangle formation might
actually protect cells by sequestering the cytotoxic oligomeric
p-tau species (20).

In vitro tau and p-tau aggregation (amy-
loidogenesis) assays will help shed light on the pathway and
pathological roles of tau fibrillization, and they will provide a
platform for high-throughput screening for tau aggregation
modulators and potential AD therapeutics (21). To date, all
tau-aggregation-based drug screens use unmodified tau (22,
23), despite the fact that the pathological tau species are
hyperphosphorylated. We posited that PIMAX-Cat would be a
viable choice for the production of p-tau more suitable for tau
fibrillization studies and AD drug discovery. To test this no-
tion, we paired tau with GSK-3β/H9252 in PIMAX-Cat and fused
these proteins to Fos and Jun, respectively. SDS-PAGE anal-
ysis of the post-induction bacterial lysates revealed high lev-
els of recombinant protein expression (Fig. 2A). Importantly,
lanes 4 and 5 in Fig. 2A show a clear GSK-3β-dependent
tau mobility shift, suggesting efficient phosphorylation (see
below).

We then assessed the efficacy of using Fos–Jun dimeriza-
tion as a protein co-purification tool. Bacterial lysates with tau
and different Fos and Jun combinations were subjected to Ni
affinity purification. The His tag was fused only to Fos. This
design ensured that all Jun fusion proteins were purified via
their persistent participation in the Fos/Jun complex, thus
increasing the likelihood of obtaining more efficiently modified
proteins and achieving stoichiometry of a heterodimer in the
CoP extension (detailed below). Lane 9 of Fig. 2A shows that
Jun–tau was effectively purified via the small His–Fos peptide
(too small to be seen in this gel). Omitting either Fos or Jun
prevented this co-purification (lanes 7 and 8), demonstrating
that Fos–Jun heterodimerization was essential and sufficient for
coupling two proteins fused to these PIMs. When GSK-3β
was fused to Fos, a single-step Ni purification resulted in nearly
stoichiometric isolation of Jun–tau and Fos–GSK-3β, with the
former exhibiting a quantitative mobility shift (lane 10). Treating
this co-purified tau protein with phosphatase restored the mo-
bility (Fig. 2B, lane 3), but not if phosphatase was inhibited by a
high concentration of phosphate (lane 4), demonstrating that
tau was very effectively phosphorylated by GSK-3β.
Importantly, all eight positive phospho-epitopes in the CDK5-generated tau phosphorylation patterns (Fig. 3B) were known GSK-3β targets. In contrast, pS422, though an AD phosphorylation site, has not been shown to be modified by GSK-3β or CDK5 (17). Comparatively, p-tau (CDK5) appeared to deviate more from the Tau Phosphorylation Database (D. Hanger, King’s College London, Institute of Psychiatry). pS199, pT205, and pS404 are listed as positive CDK5 sites but either were not detectable or varied from batch to batch in our PIMAX-Cat product (data not shown). Also, pS409 was strongly reactive here but has yet to be included in this database. Overall, five out of nine (55%) of the phospho-epitopes were in common between the PIMAX-Cat p-tau(CDK5) and the Tau Phosphorylation Database. Taking these data together, we concluded that the PIMAX-Cat-generated tau phosphorylation epitopes agreed reasonably well with previous findings.

p-Tau Produced by PIMAX Oligomerizes More Efficiently—After establishing that PIMAX-Cat was capable of producing quantitatively phosphorylated tau with GSK-3β, we tested the effect of hyperphosphorylation on tau oligomerization in vitro as a means to assess the biological relevance of this approach. In addition to p-tau generated by GSK-3β, we made a PIMAX vector expressing CDK5 (24) to produce another p-tau species. CDK5 and GSK-3β both have been linked to AD; however, it is unclear whether p-tau generated by either kinase would contribute differently to AD development via, for example, differences in aggregation kinetics. Fig. 3A shows that PIMAX-Cat generated two p-tau species with comparable purity and gel mobility retardation. Immunochemical analysis using selective antibodies against general or phosphorylated tau epitopes revealed differences in GSK-3β- and CDK5-generated tau phosphorylation patterns (Fig. 3B). Importantly, all eight positive phospho-epitopes in the p-tau(GSK-3β) species were known GSK-3β targets. In contrast, pS422, though an AD phosphorylation site, has not been shown to be modified by GSK-3β or CDK5 (17). Comparatively, p-tau (CDK5) appeared to deviate more from the Tau Phosphorylation Database (D. Hanger, King’s College London, Institute of Psychiatry). pS199, pT205, and pS404 are listed as positive CDK5 sites but either were not detectable or varied from batch to batch in our PIMAX-Cat product (data not shown). Also, pS409 was strongly reactive here but has yet to be included in this database. Overall, five out of nine (55%) of the phospho-epitopes were in common between the PIMAX-Cat p-tau(CDK5) and the Tau Phosphorylation Database. Taking these data together, we concluded that the PIMAX-Cat-generated tau phosphorylation epitopes agreed reasonably well with previous findings.

p-Tau generated via exhaustive incubation with mouse brain extract (25) or recombinant GSK-3β (26) exhibited a faster rate and higher efficiency of oligomerization in vitro than the unphosphorylated counterparts, consistent with the pathological findings linking p-tau to AD development. To compare the fibrillation behaviors of PIMAX-Cat p-tau, we subjected all three (p-)tau isoforms to in vitro fibrillation assays in the presence or absence of heparin, a common tau aggregation inducer (27). In both conditions, p-tau(GSK-3β) exhibited the fastest and most efficient oligomerization (Fig. 3C), followed by p-tau (CDK5) and then the unphosphorylated tau. Interestingly, heparin elicited comparable enhancement for all three tau species, suggesting that hyperphosphorylation and heparin induced tau aggregation via two different pathways, a finding that would potentially impact AD drug target selection.

Together, Figs. 2 and 3 demonstrate the feasibility of using PIMAX-Cat to produce hyperphosphorylated tau species for biochemical and structural studies pertaining to AD pathogenesis and drug discovery.
PIMAX-Cat to Produce Covalently Modified Tumor Suppressor p53 — To ascertain the general applicability of PIMAX-Cat, we produced acetylated and phosphorylated tumor suppressor p53 (Fig. 4A). p53 is a gatekeeper for genome stability (28). Upon genotoxic stresses, p53 is activated through multiple signaling pathways that result in a plethora of p53 PTMs. Among the most notable PTMs of p53 are acetylation and phosphorylation (29, 30). Mechanistic understanding of the molecular, anti-tumor functions of these p53 PTMs can benefit from the availability of modified p53. To this end, we fused the entire p53 open reading frame cDNA to a JunD leucine zipper. cDNAs encoding three enzymes, Aurora A kinase (full length), yeast Gcn5 (Pro19–Cys252 lacking the C’ bromodomain), and human p300 (Gln1195–Met1588, the HAT catalytic domain and the PHD domain) acetyltransferases, were then separately fused to His–FosB (Fig. 4A). Aurora A phosphorylates Ser215 within the DNA binding domain of p53 (31), whereas Gcn5 and p300 each acetylate the carboxyl-terminal regulatory domain at Lys320 (Gcn5) and Lys373/382 (p300) (29).

When co-expressed with Fos–Aurora A kinase, Jun–p53 exhibited a clear mobility shift (double asterisk, lane 6, Fig. 4B). Ni co-purification and phosphatase treatment caused this p53 species to regain its normal mobility, confirming that Aurora A phosphorylated p53 well within the PIMAX-Cat context (top panel, Fig. 4D). In stark contrast, GSK-3β failed to change the mobility of p53 (data not shown) even though it modified tau very effectively (Figs. 2 and 3). Also important was that Aurora A required the concomitant presence of Fos and Jun to phosphorylate p53 (lanes 4 and 5, Fig. 4B), suggesting that, unlike GSK-3β, Aurora A by itself was unable to catalyze significant p53 phosphorylation. Intriguingly, immunoblotting with a commercial Ab-1 monoclonal antibody that recognized the p53 peptide212FRHSVV217 failed to detect the phosphorylated p53 (lane 2, bottom panel, Fig. 4C). Treating the Ni-NTA-purified phosphorylated p53 with phosphatase restored Ab-1 reactivity (Fig. 4D), strongly suggesting that Ser215 was likely phosphorylated to completion by Aurora A within the PIMAX-Cat context (31).

We next examined p53 acetylation by co-expressing the catalytic domain of yeast Gcn5 and human p300 acetyltransferases. The catalytic domain of yeast Gcn5 shares more than 80% identity with its two human homologues, GCN5 (KAT2A) and PCAF (KAT2B) (data not shown). Gcn5 acetylates Lys320, whereas p300 prefers Lys373 and Lys382 (32–34). Bacterial
lysates containing p53 and different enzymes were examined via immunoblotting. Gcn5 in PIMAX-Cat acetylated Lys320 and Lys373/382 (lane 3), whereas p300 acted specifically on Lys373/382 (lane 4, Fig. 4E). These results in general agreed with the reported p53 specificities for Gcn5 and p300, except that Lys373/382 acetylation by human Gcn5 has not been reported. This discrepancy might have resulted from the sequence differences between yeast and human Gcn5 homologues.

From the data presented in Figs. 2 to 4, it is clear that PIMAX-Cat can generate multiple proteins bearing different PTMs at high efficiencies. Furthermore, the modular design of the vectors allows each of the enzymes and substrates to be easily coupled to additional PIMAX-Cat partners, making future comprehensive studies of these PTMs (e.g. all potential substrates of GSK-3β/H9252, all p53 PTMs, etc.) possible.

**PIMAX-CAF Increases Solubility of p53**—Conceptually, any two proteins can be brought to proximity by PIMs such as Fos and Jun. The use of PIMAX should thus cover beyond the production of posttranslationally modified proteins. Here we tested two other extensions of PIMAX: CAF and heterodimer CoP.

PIMAX-CAF integrates a molecular chaperone to help improve the solubility and hence the yield of recombinant proteins. Specifically, we chose TcFKBP18, an FK506-binding protein from the hyperthermophilic archaeon *Thermococcus* sp. KS-1 (35). Unlike other multicomponent *E. coli* protein-folding machinery such as DnaJ/DnaK and GroEL/GroES, TcFKBP18 acts alone to trap and refold denatured proteins in vitro and can increase the solubility of several recombinant proteins in vivo (13) when fused physically to these clients. Taking advantage of the small size of TcFKBP18, as well as the simplicity of the system’s setup, we tested whether this molecular chaperone can facilitate protein solubility in the context of PIMAX-CAF.

To establish a TcFKBP18-based PIMAX-CAF system, we used p53 as the client (Fig. 5A). Recombinant full-length p53 typically was highly insoluble in our hands (lanes 2 and 3, Fig. 5B). The TcFKBP18 open reading frame was fused to Fos in a plasmid that already contained the Jun–p53 chimera. After standard isopropyl β-D-1-thiogalactopyranoside induction at 37 °C, we assessed p53 solubility by means of centrifugation and SDS-PAGE. The solubility of p53 was drastically improved by TcFKBP18 (lanes 4 and 5, Fig. 5B), agreeing well with the reported ability of this molecular chaperone to trap and refold many heterologous misfolded recombinant proteins (13, 35). Further testing of the power and versatility of TcFKBP18 was done in the next application, PIMAX-CoP.

**PIMAX-CoP for the Purification of Active CDK5/p25 Kinase Complex**—The PIMAX-Cat and -CAF applications described above involved protein pairs that interact transiently. However, many proteins function within a dimer. Biochemical reconstitution of such dimeric complexes by separately preparing individual subunits increases the labor and cost. We posited that Fos and Jun dimerization could promote the assembly and purification of dimeric protein complexes. To
test this hypothesis, we expressed a cyclin-dependent kinase complex, CDK5/p25.

Cyclin-dependent kinases are essential for numerous cellular functions, and CDK dysfunction can have catastrophic consequences (36). Human CDK5 plays critical roles in brain development, synaptic plasticity, behavior, and cognition (24). Normal CDK5 associates with an activator, p35 or p39 (37). In AD patients, CDK5 instead complexes with p25, which is derived from p35 proteolysis (38, 39). Associating with p25 is thought to hyperactivate CDK5 in AD patients, but the underlying molecular mechanism remains enigmatic (40).

To express and purify the CDK5/p25 complex via PIMAX-CoP, we inserted the cDNA of the entire CDK5 coding sequence and a fragment from p35 that corresponded to the disease-associated p25 polypeptide (i.e. Ala99 to Arg307) into a PIMAX vector. This vector had additional modifications to meet the needs of the CoP extension (Fig. 6A). Firstly, in addition to the His tag in front of Fos, a FLAG tag was introduced to the Jun fusion protein. Successive Ni-NTA and anti-FLAG affinity chromatography purification would ensure stoichiometry. Secondly, a TEV protease site was included in both recombinant proteins, so that a single TEV reaction step could liberate both subunits. Finally, the TcFKBP18 molecular chaperone used for PIMAX–CAF (Fig. 5) was also included as an optional aid for protein solubility improvement.

Bacterial lysates were processed through sequential affinity purification and examined via SDS-PAGE (Fig. 6B). We noticed that both CDK5 (labeled with a pound symbol) and p25 (marked with an asterisk) were low in solubility (lanes 2 and 3). However, when TcFKBP18 was included as part of the CDK5 fusion protein (but not an integral part of the p25 recombinant protein), the expression level and solubility of both recombinant proteins were improved (lanes 4 and 5), indicating that TcFKBP18 functioned well both in cis and in trans. To isolate the CDK5/p25 complex, we applied cell lysates to a Ni-NTA column to trap the His-tagged Fos–FKBP–CDK5 protein. Jun–p25 fusion, in a complex with Fos–CDK5, would be co-purified as well. Imidazole eluted both proteins, even though only one of the two proteins had the His tag. However, impurities were obvious in the imidazole eluates. To further enrich for the CDK/p25 heterodimer, we passed imidazole eluates through an anti-FLAG affinity column, and we eluted the bound CDK5/p25 dimer with a glycine wash (lane 6, Fig. 6C) or on-bead TEV digestion (lane 7, Fig. 6C). Both methods liberated CDK5/p25 from the anti-FLAG column, but TEV-digestion products appeared to be cleaner. The His-tagged recombinant TEV was further removed by passing the mixture through another round of Ni-NTA binding (data not shown). The final PIMAX-CoP product was then used in an in vitro phosphorylation reaction to examine its enzymatic activity. The substrate was recombinant tau protein literally identical to that shown in Fig. 3. Within one hour of incubation, we observed a significant mobility shift of tau incubated with the CDK5/p25 complex (Fig. 6D). Tau phosphorylation seemed to reach a maximum in 4 h. We thus concluded that PIMAX-CoP effectively produced an enzymatically active CDK5/p25 complex.

**DISCUSSION**

Conserved protein–protein interaction modules provide a platform for the execution and regulation of numerous protein functions. Here we report the exploitation of one of the best-studied PIMs for the production of several proteins that have proven to be challenging to synthesize in *E. coli*. This PIMAX approach is based on the fact that many proteins require transient (e.g. PTM and chaperone-facilitated folding) or stable interaction with another facilitator protein; co-expression of these two partner proteins frequently results in only partial success because the bacterial cellular environment may not permit the most efficient interaction between the two subject proteins. The high-affinity, heterospecific Fos5 and JunD leucine zipper domains (41) provide a microenvironment that facilitates the association of two proteins fused to these zippers.

Pernelle et al. (42) reported the dissociation constant between Fos and Jun leucine zippers as $110 \pm 12$ nM in vitro. Although it is not clear whether this can be directly translated to the Fos–Jun interaction taking place in *E. coli* cells such as those reported in this work, our observations that very little Jun–tau was present in the flow-through fraction of Ni-affinity chromatography of His–Fos fusion proteins (data not shown) indicate very strongly that the majority of Fos- and Jun-fusion proteins were part of the complex and could be co-purified. It should be noted, though, that the relative amounts of our Fos- and Jun-fusion proteins varied from application to application.
The maximal yield of the recombinant protein complex thus was dictated by the one expressed at the lower level. There have been multiple attempts to produce recombinant proteins bearing a posttranslational modification by simply co-expressing the enzyme and substrate proteins (43–46). We and others previously developed the tethered catalysis/yeast two-hybrid system and one of its derivatives for bacterial expression of posttranslationally modified proteins (47–49). Tethered catalysis differs from other co-expression methods in that the enzyme is fused to its substrate, ensuring physical linkage of the two. Though very efficient and convenient, the construction of some tethered catalysis recombinant genes can be challenging because of the large size of certain enzymes and substrates. Also, the spatial arrangement of the pair can be more restrictive, thus interfering with or even preventing catalysis (data not shown). The PIMAX approach relies on the strong and specific heterodimerization of Fos and Jun leucine zippers to bring two proteins of interest together. The modular, bipartite design allows easier plasmid construction. It should be noted that Fos and Jun leucine zippers interact in a parallel fashion (50), and thus may also restrict the interaction of certain target protein pairs. Potential remedies for such an issue include the use of anti-parallel PIMs (51) and moving Fos/Jun to the carboxyl terminus of the recombinant gene. Next-generation PIMAX vectors with Fos and Jun each sandwiched by two different LIC boxes will quadruple the cloning possibilities.

Despite the very efficient modifications of tau and p53 as shown in Figs. 2 through 4, we have yet to definitively construct a comprehensive modification “map” of these proteins. It remains to be determined whether the multiple phospho-serine and -threonine sites detectable by antibodies (Fig. 3B) co-existed in one p-tau molecule or were distributed through...
multiple populations. In a similar PIMAX-Cat setup, we generated different modified histone H3 (data not shown and Ref. 52). The histone acetyltransferase Gcn5 acetylated H3 effectively and left practically no unacytated species (52). However, the acetylated H3 contained from one to seven acetates per molecule. The heterogeneity seems to suggest that even with the aid of Fos-Jun heterodimerization, these enzymes are not forced to conduct totally promiscuous catalysis. Indeed, GSK-3β and CDK5 apparently generated different tau phosphorylation patterns (Fig. 3B); GSK-3β did not cause a p53 mobility shift (data not shown), and Gcn5 and p300 acted on different lysine residues of p53 (Fig. 4E).

Given its ease of operation and modular nature, we suggest that the PIMAX approach has significant potential in the proteomic field. Echoing an early suggestion that Fos and Jun leucine zipper domains be used as a molecular Velcro in biotechnology (53) are studies that utilized leucine zippers to verify the substrate for a tyrosine kinase (54), to facilitate the amyloid formation of α-synuclein (55), to enhance the interaction of different proteins in a yeast two-hybrid setup (56), and to help design synthetic leucine zipper peptides that exhibit extremely high affinity for heterodimerization (41, 57–59). This work presents a suite that incorporated Fos and Jun to solve different challenges faced in recombinant protein expression and purification. In addition to Fos and Jun, we have developed a different protein interaction module taking advantage of the extensive intramolecular interactions of the SUMO protein. With these existing and novel PIMs, general and task-specific applications of PIMAX are envisioned.

Besides the three PIMAX extensions introduced in this work, proteomic tools that take advantage of certain PIMs can be developed. For example, Zhang et al. incorporated two artificial leucine zipper domains into their protein chips as a means of protein immobilization (60). In this work, one of two matching zippers was fixed to a glass slide and then used to trap recombinant proteins fused to the complementary zipper. Following this design, we suggest that a “PIMAX-Cat on a protein chip” approach can be developed to help identify protein substrates for a given enzyme at a higher efficiency. Those enzymes with relatively low affinity, with high on/off rates, or requiring a cofactor (e.g. cyclin for CDKs) might now become more useful in microarray screens (61).

Lastly, the capability of PIMAX can be further expanded if new, higher capacity PIMs are obtained. Current PIMAX approaches using Fos and Jun leucine zippers are limited to binary interactions. Higher ordered interactions could be achieved if trimeric or even tetrameric PIMs were developed. For example, Deng et al. reported that two mutant GCN4 leucine zippers form anti-parallel heterospecific tetramers (62). These and similar PIMs might help researchers realize PIMAX applications involving more than two proteins, including enzymes with an obligatory co-factor, and trimeric or tetrameric protein complexes.

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