Protein Expression and Purification **19,** 312–318 (2000)
doi:10.1006/prep.2000.1251, available online at http://www.idealibrary.com on IDEAL®

Controlled Intracellular Processing of Fusion Proteins by TEV Protease

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Received March 13, 2000, and in revised form March 31, 2000

Here we describe a method for controlled intracellular processing (CIP) of fusion proteins by tobacco etch virus (TEV) protease. A fusion protein containing a TEV protease recognition site is expressed in *Escherichia coli* cells that also contain a TEV protease expression vector. The fusion protein vector is an IPTG-inducible ColE1-type plasmid, such as a T7 or *tac* promoter vector. In contrast, the TEV protease is produced by a compatible p15A-type vector that is induced by tetracyclines. Not only is the TEV protease regulated independently of the fusion protein, but its expression is highly repressed in the absence of inducer. Certain fusion partners have been shown to enhance the yield and solubility of their passenger proteins. When CIP is used as a purification step, it is possible to take advantage of these characteristics while both eliminating the need for large amounts of pure protease at a later stage and possibly simplifying the purification process. Additionally, we have observed that in some cases the timing of intracellular proteolysis can affect the solubility of the cleaved passenger protein, allowing it to be directed to either the soluble or the insoluble fraction of the crude cell lysate. This method also makes it possible to quickly gauge the efficiency of proteolysis *in vivo*, before protein purification has begun and *in vitro* processing is attempted. © 2000 Academic Press

Genetically engineered affinity tags are often exploited to facilitate the expression and purification of recombinant proteins (1,2). Not only do they make protein purification more efficient, but affinity handles such as maltose-binding protein (MBP)[2] (3) and glutathione *S*-transferase (4) have also been observed to protect passenger proteins from intracellular proteolysis and enhance their yield (5–7). Furthermore, MBP has been demonstrated to increase the solubility of its fusion partners (8,9). However, due to concerns about the effect of a large affinity tag on the structure and activity of a passenger protein, it is usually desirable to obtain the native protein free from the affinity handle.

Typically, the passenger protein is separated from its fusion partner by site-specific proteolysis after affinity chromatography. In some circumstances, however, the ability to cleave fusion proteins inside intact cells would be very useful. For example, it is relatively common to encounter a fusion protein that cannot be processed effectively because of steric hindrance at the cleavage site. Coexpression of the protease with its fusion protein substrate would allow problems of this nature to be detected at a very early stage, before purification has begun. Not only would this detection save time, but the information could also be used to prioritize projects when the number of fusion proteins to be processed exceeds the available resources (e.g., the supply of purified protease). Another recurring problem is that affinity-tagged fusion proteins occasionally fail to interact efficiently with their immobilized ligands (8,10). Because the affinity tag would offer little assistance during purification in these circumstances, considerable savings in both time and money could be realized by cleaving these problematic fusion proteins *in vivo*. Finally, regardless of whether or not one intends to use the affinity handle for purification, intracellular processing might also provide an early indication of how a passenger protein will behave after it is cleaved from its fusion partner. If the pas-

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[2] Abbreviations used: MBP, maltose-binding protein; CIP, controlled intracellular processing; TEV, tobacco etch virus; aTet, anhydrotetracycline hydrochloride; ORF, open reading frame; PCR, polymerase chain reaction; GFP, green fluorescent protein; TIMP, the N-terminal inhibitory domain of human tissue inhibitor of metalloproteinases-2.

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senger protein tends to form insoluble aggregates *in vivo*, the chances are good that this would also occur *in vitro*.

Here we describe a method, termed controlled intracellular processing (CIP), for using the catalytic domain of tobacco etch virus (TEV) protease to cleave fusion proteins in *Escherichia coli* cells. The source of TEV protease is an expression vector that is compatible with most commonly used fusion protein expression vectors (e.g., pMal-C2 (New England Biolabs), pThio-His (Invitrogen), pGEX (Amersham-Pharmacia), pET (Novagen)). For added flexibility, the TEV protease vector is tetracycline-inducible, which makes it possible to regulate its production independently of an IPTG-inducible fusion protein substrate, a noteworthy feature distinguishing this system from one described previously (11).

To evaluate the efficacy of the CIP system, we examined the intracellular processing of four different fusion proteins. All four substrates were cleaved with high efficiency *in vivo*. Additionally, the solubility of two of the passenger proteins was improved by delaying the timing of intracellular processing, illustrating the advantage of an independently regulated protease vector.

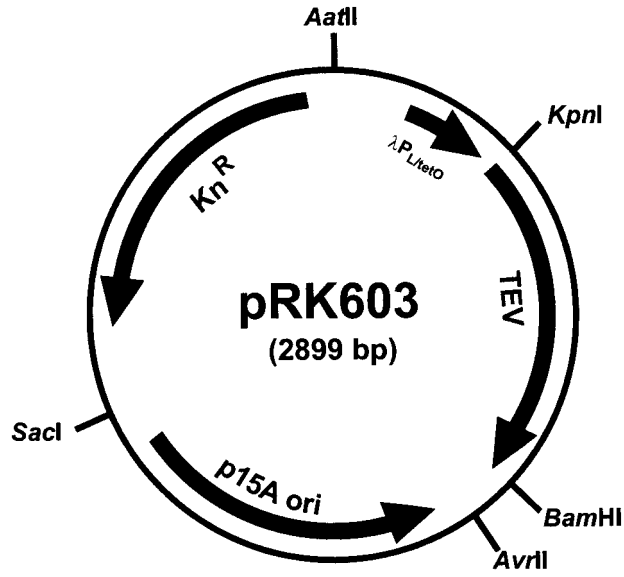

FIG. 1. Schematic map of the TEV protease expression vector pRK603. The unique restriction sites used for subcloning are indicated. Kn^R, kanamycin resistance gene; TEV, TEV protease open reading frame; $\lambda P_{L/tetO}$, synthetic phage lambda P_L promoter and *tet*O2 operator combination (12); p15A ori, p15A origin of replication.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, and Deep Vent DNA polymerase were obtained from New England Biolabs (Beverly, MA). Synthetic oligodeoxyribonucleotides were purchased from BioServe Biotechnologies (Laurel, MD) or Life Technologies, Inc. (Rockville, MD). IPTG was obtained from 5 Prime → 3 Prime, Inc. (Boulder, CO), and anhydrotetracycline hydrochloride (aTet) was purchased from Fisher Scientific (Pittsburgh, PA).

Plasmid Expression Vectors

The following bacterial strain and plasmids were gifts from Dr. Hermann Bujard: DH5αZ1, pZE21-MCS1, pZS*24-MCS1, and pZA31-Luc (12). The DH5αZ1 strain has constitutively active Tet repressor (TetR) and Lac repressor (LacI) genes integrated, in tandem, into the bacterial chromosome on a lambda prophage. To construct pRK603, a 237-amino-acid open reading frame (ORF) encompassing the TEV protease catalytic domain was amplified from a TEV protease expression vector, similar to one described previously (9), by polymerase chain reaction (PCR) with the following primers: PE-137, 5′-ATTATGGTACCATGGGAGAAAGCTTGTTTAAGG-3′ (overlaps with the N-terminus of the TEV protease ORF; the *Kpn*I site immediately preceding the initiator Met codon is underlined); and PE-30, 5′-GCAAGGCGATTAAGTTGGGTAACGC-3′ (anneals to the vector downstream of the TEV protease C-terminus). The PCR product was cleaved with *Kpn*I and *Bam*HI (the *Bam*HI site was present in the template, immediately after the C-terminus of the TEV protease ORF) and then ligated with the *Kpn*I/*Bam*HI vector backbone of pZE21-MCS1 to create pRK558. Next, the region containing the $\lambda P_{L/tetO}$ promoter and the TEV protease ORF was removed from pRK558 with *Aat*II and *Avr*II and ligated with the *Aat*II/*Avr*II vector backbone of pZS*24-MCS1 to construct pRK586. Finally, the *Sac*I/*Avr*II fragment containing the pSC101* origin of replication (*ori*) was replaced with the corresponding fragment from pZA31-Luc containing the p15A *ori*, yielding pRK603 (Fig. 1).

To construct fusion protein substrates for TEV protease, the passenger protein ORFs were amplified by PCR from MBP fusion vectors (9). A TEV protease recognition site (ENLYFQG) was added to the 5′ end of the forward PCR primer so that after intracellular processing, the P1′ glycine residue from the TEV protease recognition site would be the first amino acid at the N-terminus of the passenger protein (Fig. 2).

Protein Expression and SDS–PAGE Analysis

Cells from single drug-resistant colonies were grown overnight in LB broth (13) supplemented with the appropriate antibiotic(s) (100 μg/ml ampicillin and/or 30 μg/ml kanamycin) at 37°C. The next morning, the cells were diluted 1:50 in the same medium and grown in

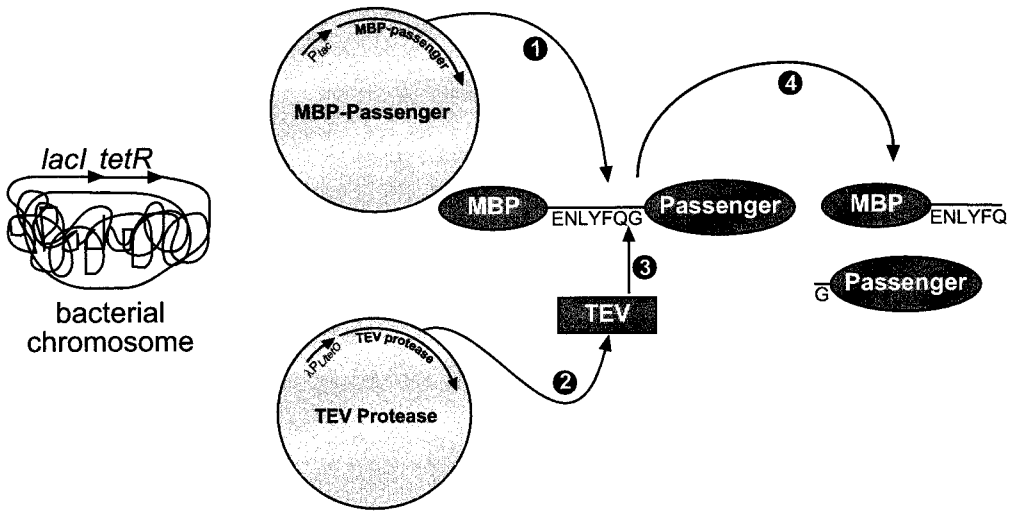

FIG. 2. Strategy for controlled intracellular processing of fusion proteins by TEV protease. The host strain, DH5αZ1, has genes encoding the regulatory proteins tetR and lacI integrated in tandem into the bacterial chromosome (12). The fusion protein (MBP-Passenger) and TEV protease (TEV) are synthesized from separate compatible plasmid expression vectors that can be regulated independently of one another (steps 1 and 2, respectively). Upon induction with IPTG and aTet, the protease cleaves the fusion protein at the designed site (step 3), yielding separate MBP and passenger protein moieties (step 4). P_{tac}, *tac* promoter; $\lambda P_{L\text{-}tetO}$, synthetic, tetracycline-inducible promoter/operator combination (12); tetR, tetracycline repressor gene; lacI, *lac* repressor gene; ENLYFQG, TEV protease recognition site.

shake flasks to early log phase (A_{600} = 0.3–0.5) at 37°C, at which time the temperature was shifted to 30°C and IPTG was added to a final concentration of 1 mM to initiate production of the fusion protein. To induce TEV protease expression in DH5αZ1 cells, aTet was added to a final concentration of 100 ng/ml either at the same time as the addition of IPTG or 2 h later, as indicated. After 4 h at 30°C, the cells from 10 ml of each culture were recovered by centrifugation and resuspended in 1 ml of 50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl. The cells were lysed by sonication, and total protein and soluble extract samples were collected as described (9). Samples were analyzed on 10–20% SDS–polyacrylamide gels (Novex, San Diego, CA) and visualized by staining with GelCode Blue (Pierce, Rockford, IL).

RESULTS

Construction of the TEV Protease Expression Vector pRK603

The main objective of this study was to devise a reliable and efficient method for site-specific proteolysis of proteins in intact cells. We chose TEV protease for this purpose because of its high degree of sequence specificity (14) and because it could be expressed in an active form in the *E. coli* cytoplasm without interfering with cell viability (11). To maintain as much control as possible over the system, we wanted to be able to regulate production of the enzyme and substrate independently of one another. Therefore, we needed to construct a TEV protease vector that would be compatible with ColE1-type replicons, which are commonly used to produce fusion proteins, and that could be induced by a compound other than IPTG.

Although a variety of methods have been used to regulate the expression of heterologous genes in *E. coli* (16–21), we were especially interested in chemical inducers because they are the most versatile. Several arabinose-inducible (P_{BAD}) vectors have been described (22–24); however, the original claim that the P_{BAD} promoter is very tightly regulated has lately been challenged (25). Lutz and Bujard recently described a series of vectors that utilize a synthetic promoter/operator combination to achieve tightly regulated protein expression (12). In these vectors, the very strong $\lambda P_{L/tetO}$ promoter is controlled by tandem copies of the tetR operator site, resulting in a promoter that can be induced by tetracyclines, the most useful being aTet. Not only does aTet have a higher binding constant for tetR than tetracycline, but it also has a lower antibiotic activity (12,26). The modular nature of the Lutz/Bujard plasmids makes it relatively easy to assemble a vector with the desired properties of antibiotic resistance, plasmid compatibility, and copy number. For these reasons, we decided to try the $\lambda P_{L/tetO}$ vectors.

Most of the commonly used plasmid expression vectors are derivatives of the ColE1 replicon. To coexist stably with such plasmids, the TEV protease expression vector needs to be a member of a different compatibility group (27). Initially, we amplified the coding sequence of the TEV protease catalytic domain by PCR

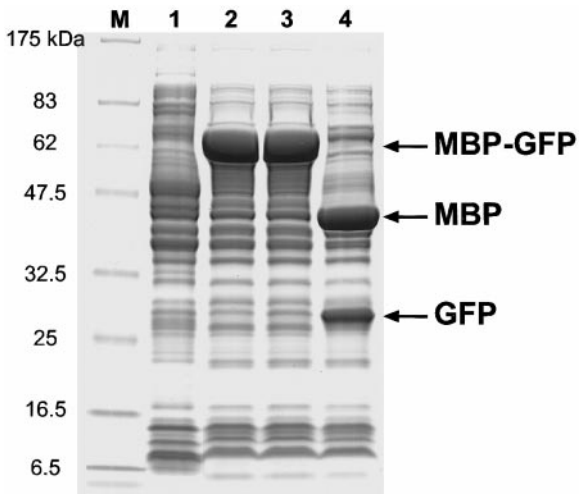

FIG. 3. Controlled intracellular processing of an MBP–GFP fusion protein by TEV protease. DH5αZ1 cells containing either pRK603 or the MBP–GFP vector were induced with IPTG (lanes 1 and 2, respectively). Cells containing both plasmids were induced with IPTG only (lane 3) or both IPTG and aTet (lane 4). Samples of the total intracellular protein were resolved by SDS–PAGE (10–20% Tris–glycine gradient gel (Novex)) and stained with GelCode Blue (Pierce). M, broad-range molecular weight marker (New England Biolabs).

and ligated this sequence with pZE21-MCS1. However, this plasmid is a member of the ColE1 compatibility group. Therefore, through an intermediate construct, we eventually obtained pRK603 (Fig. 1), a kanamycin-resistant p15A-type vector with the $\lambda P_{L/tetO}$ promoter.

TEV Protease Expression Is Tightly Regulated in DH5αZ1 Cells

Our first goal was to determine whether the TEV protease gene in pRK603 is tightly regulated. This experiment was performed in DH5αZ1 cells, which constitutively synthesize TetR (12). The substrate was a fusion protein composed of MBP and *Aequorea victoria* green fluorescent protein (GFP) (28), with a canonical TEV protease recognition site in the linker between the two domains. Four hours after IPTG induction, we compared the composition of the total intracellular protein from DH5αZ1 cells containing only the MBP–GFP fusion protein vector with that from cells also containing the TEV protease expression vector pRK603 (Fig. 3, lanes 2 and 3, respectively). The two samples were indistinguishable from each other, illustrating that the production of TEV protease was completely repressed when aTet was not added to the cultures. Upon induction of pRK603 with aTet, the fusion protein was processed almost entirely to yield separate MBP and GFP moieties (Fig. 3, lane 4). Thus, although the fully induced $\lambda P_{L/tetO}$ vector did not produce enough TEV protease to be readily detected by SDS–PAGE, evidently enough of the protease was made to process an abundant fusion protein to near completion *in vivo*.

Constitutive Expression of TEV Protease Affects Neither the Yield nor the Processing of the Fusion Protein

One of the more commonly used strains of *E. coli* for protein expression is BL21/DE3 (16); however, this strain does not produce TetR. In these circumstances, the expression of TEV protease would be constitutive, and we were concerned that this would affect the cell viability, yield of fusion protein, and/or efficiency of processing. At the same time, we wanted to investigate whether other fusion proteins besides MBP–GFP could also be processed efficiently in cells containing pRK603. Therefore, we fused three additional passenger proteins to the C-terminus of MBP: TIMP (the N-terminal inhibitory domain of human tissue inhibitor of metalloproteinases-2) (29), p16 (human cyclin-dependent kinase 4 inhibitor) (30), and E6 (an oncoprotein encoded by human papillomavirus 18) (31). Like MBP–GFP, each of these fusion proteins contained a canonical TEV protease recognition site in the linker between the domains. Nearly complete processing of all four fusion proteins occurred in BL21/DE3 cells (Fig. 4). Moreover, we noted that the yield of GFP was very similar in BL21/DE3 and DH5αZ1 cells (compare Figs. 3 and 4). Thus, these results demonstrated that the TEV protease vector pRK603 can be used in cells other than DH5αZ1, even when expression of TEV protease is constitutive, with no detrimental effect on

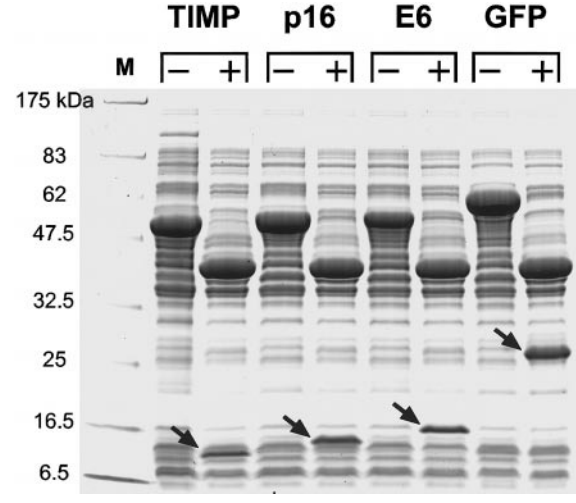

FIG. 4. Intracellular processing of fusion proteins in *E. coli* strain BL21/DE3. Cells containing one of the MBP fusion vectors (MBP–TIMP, MBP–p16, MBP–E6, or MBP–GFP, as indicated), either alone (−) or together with pRK603 (+), were grown to mid-log phase at 37°C and then induced with IPTG for 4 h at 30°C. Samples of the total intracellular protein were resolved by SDS–PAGE (10–20% Tris–glycine gradient gel, Novex) and stained with GelCode Blue (Pierce). M, broad-range molecular weight marker (New England Biolabs). Arrows mark the positions of the cleaved passenger proteins.

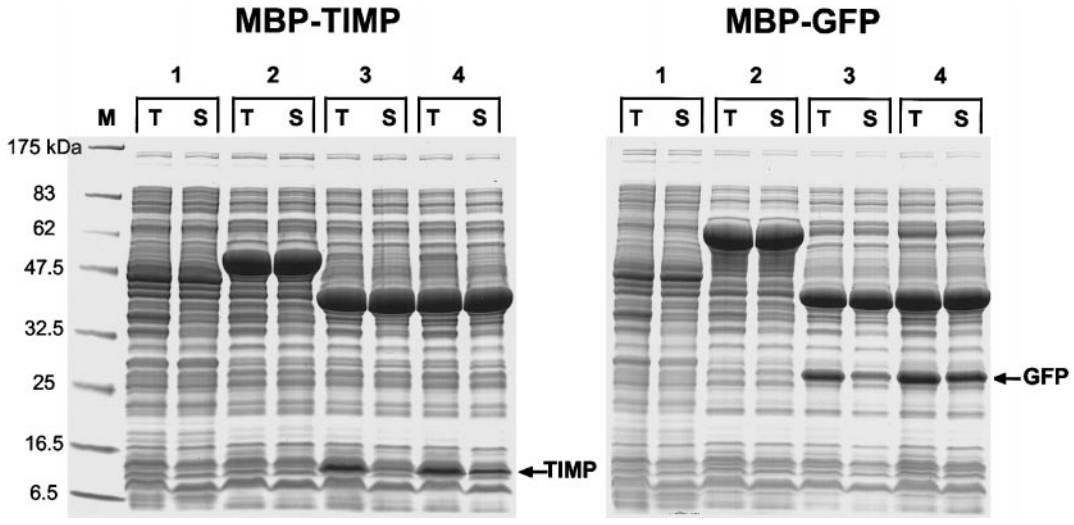

FIG. 5. The solubility of TIMP and GFP is affected by the duration of their association with MBP. DH5αZ1 cells containing pRK603 only (lane 1), either the MBP–TIMP or the MBP–GFP expression vector only, as indicated (lane 2), or both plasmids (lanes 3 and 4) were induced with IPTG at mid-log phase, at which point the temperature was reduced from 37 to 30°C for the duration of the experiment. Cells containing both plasmids (lanes 3 and 4) were also induced with aTet, either at the same time (lane 3) or 2 h after IPTG induction (lane 4). All cultures were harvested 4 h after IPTG induction. Samples of the total (T) or soluble (S) intracellular proteins were resolved by SDS–PAGE (10-20% Tris–glycine gradient gel, Novex) and stained with GelCode Blue (Pierce). M, broad-range molecular weight marker (New England Biolabs). The positions of the TIMP- and GFP-cleaved passenger proteins are indicated.

either the yield of the fusion protein or the efficiency of processing.

Delayed Processing Can Improve the Solubility of Some Passenger Proteins

When the MBP fusion proteins that were used in the previous experiments were cleaved right away (e.g., in BL21/DE3 cells), all four of the passenger proteins accumulated predominantly in an insoluble form (data not shown). This result was not surprising, because these proteins are insoluble when produced in an unfused form in *E. coli* (9). Therefore, it would seem they are protected from aggregation only as long as they are fused to MBP. However, three of these passenger proteins remained soluble when they were processed by TEV protease *in vitro,* after affinity chromatography (data not shown). This result prompted us to examine whether the solubility of some passenger proteins could be influenced by the duration of their association with MBP. Interestingly, we discovered that this is indeed the case; when the induction of TEV protease was delayed until 2 h after IPTG was added to induce fusion protein expression, we observed a modest increase in the amount of soluble TIMP and a substantial increase in the amount of soluble GFP (Fig. 5). With the other two passenger proteins, no improvement in solubility was observed (data not shown).

DISCUSSION

Through the use of our CIP system in DH5αZ1 cells, which constitutively produce tetR, enough TEV protease can be synthesized upon induction with aTet to process a very abundant fusion protein to near completion *in vivo.* In contrast, no processing occurs in the absence of the inducer. We have observed a similar degree of repression in cells that contain a Tn10 transposon insertion in the chromosome (data not shown). Furthermore, even when the TEV protease production is constitutive (e.g., no TetR in the cells), neither the yield of the fusion protein, the cell viability, nor the proteolytic processing is affected. Thus, efficient processing is possible in a variety of genetic backgrounds.

The very tight regulation of the TEV protease expression vector pRK603 makes it possible to control the timing of intracellular processing. We observed that the solubility of some passenger proteins (TIMP and GFP) was markedly improved by delaying *in vivo* processing of the MBP fusion proteins until 2 h after their synthesis was induced with IPTG. In contrast, there was no improvement in the solubility of the other two passenger proteins examined in this study under the same conditions (E6 and p16). All of these passenger proteins are insoluble when expressed in an unfused form in *E. coli,* but are efficiently solubilized when fused to MBP (9). The significance of these observations is not entirely clear. We do not know, for example, whether the increase in solubility that we observed is correlated with the acquisition of native structure, or whether a similar effect can also be elicited by other soluble fusion partners. Nevertheless, our results suggest that in some cases it is possible to exploit the beneficial effect of MBP on the solubility of its fusion

partners while still avoiding the necessity of *in vitro* processing. In the same vein, we have found CIP to be a useful diagnostic tool for assessing whether the propensity of a recombinant protein to aggregate is masked only temporarily by fusing it to MBP. Passenger proteins that exhibit very poor solubility even after delayed processing *in vivo* are likely to behave similarly after processing *in vitro.*

It is possible to envision CIP as an integral part of a generic strategy for protein expression and purification. In particular, this method would be extremely useful when the passenger proteins are produced in the form of an "affinity sandwich" (32–34), bracketed by a large fusion partner like MBP and a small peptide extension such as a His-tag. The benefits of these tags are additive and, to a certain degree, complementary. For example, MBP can promote the solubility of its fusion partners, but the His-tag cannot. Conversely, the His-tag works well under denaturing conditions, but MBP does not. Because MBP must be fused to the N-terminus of a passenger protein in order to enhance its solubility (35), the C-terminus of the passenger protein is the most logical location for the His-tag. The TEV protease recognition site would be incorporated in the linker between the large MBP domain and the passenger protein to allow for separation of the two domains. In most cases, the presence of the His-tag on the C-terminus of the passenger protein would be of no concern, because its small size makes it unlikely to interfere with either structure or activity.

Working with a passenger protein in this affinity sandwich configuration, one would have the option of using sequential affinity chromatography steps to obtain very pure preparations of the fusion protein, in which case removal of the N-terminal affinity tag would be performed *in vitro.* As discussed above, CIP could still be a useful diagnostic tool in such cases. Alternatively, there are circumstances in which it may be more efficient to process the fusion protein *in vivo.* One could still exploit the favorable influence of the N-terminal fusion partner on the yield and possibly also the solubility (see above) of the passenger protein while relying solely on the His-tag, present on the C-terminus of the cleaved passenger protein after intracellular processing, to facilitate its purification. If the passenger protein is pure enough after immobilized metal-ion affinity chromatography, then the extra steps that would be needed to process a fusion protein *in vitro* and separate the cleaved product from the protease and other by-products can be avoided altogether without compromising either the purity or yield. This approach would also help allay any concerns about residual contaminating TEV protease in preparations of the target protein, which might lead to the loss of biological activity over time. CIP would also be preferable when the N- terminal fusion partner fails to interact efficiently with its immobilized ligand, as has been reported for some MBP fusion proteins (10,11). In the event that the passenger protein is insoluble after *in vivo* processing, the His-tag could still be used to purify it under denaturing conditions, if desired.

In summary, we have developed an intracellular processing expression system for cleaving fusion proteins with TEV protease in *E. coli.* The expression of the protease is tightly regulated and the processing is site specific. We have found that CIP can be a very useful diagnostic tool, often resulting in considerable savings in time and money. Moreover, CIP provides an extra measure of versatility as one element of a generic strategy for protein expression and purification.

ACKNOWLEDGMENTS

We thank Anne Arthur for expert editorial assistance and our colleagues for helpful comments and critical reading of the manuscript.

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