

# 17

## Tandem Affinity Purification to Enhance Interacting Protein Identification

Bertrand Séraphin,<sup>1,2</sup> Oscar Puig,<sup>1</sup> Emmanuelle Bouveret,<sup>1</sup>  
Berthold Rutz,<sup>1</sup> and Friederike Caspary<sup>1</sup>

<sup>1</sup>EMBL, D-69117 Heidelberg, Germany; <sup>2</sup>CGM, CNRS, F-91198 Gif sur Yvette, France

### INTRODUCTION, 313

Tag selection and design, 315

### OUTLINE OF PROCEDURE, 318

Construction of cells or organisms expressing a TAP-tagged protein, 319

Preparation of extracts, 319

Tandem affinity purification, 320

Analysis of the purified material, 321

Controls and verification, 321

### PROTOCOLS, 322

Preparation of extracts to purify the yeast Lsm3 protein, 322

Dissecting protein interactions with the TAP method, 324

### TROUBLESHOOTING AND LIMITATIONS, 326

### REFERENCES, 327

## INTRODUCTION

With the number of complete genome sequences increasing at an ever-faster pace, we are getting sequence data for the full complement of proteins or “proteome” (Wasinger et al. 1995; Wilkins et al. 1996), encoded by various organisms. To understand how the proteome confers specific properties to a host cell, one needs to obtain information regarding several parameters, including the level of expression of all proteins, their posttranslational modifications, their cellular localizations, and their interactions with different partners (proteins, nucleic acids, etc.). This task is often complicated by the wide variety of expression levels of different proteins in a given cell: The steady-state level of cellular proteins may differ by several orders of magnitude even though they are encoded by genes approximately equally represented in the genome (Gygi et al. 2000). In recent years, mass spectrometry has become a powerful tool for protein identification at an

unequaled level of sensitivity (Wilm et al. 1996). This strength has been exploited to analyze complex protein mixtures fractionated on two-dimensional gels (Shevchenko et al. 1996). However, it has become clear that only a fraction of the total cellular proteins, representing the most highly expressed polypeptides, can be detected using this strategy (Gygi et al. 2000).

Given its high sensitivity and the possibility of processing numerous samples, mass spectrometry (see Chapter 12) is also well adapted for the identification of subunits of protein complexes or interacting protein partners (Lamond and Mann 1997). The limiting step for this application of mass spectrometry resides in the difficulties encountered to obtain sufficient amounts of purified complexes. Several strategies have been successful for the purification of such protein complexes. Standard biochemical methods can be used and will remain essential for some applications (Deutscher 1990). They are, however, often cumbersome and time-consuming because optimal purification steps have to be determined empirically for each complex. Furthermore, a specific, reliable and, if possible, rapid assay needs to be designed for each new purification (activity assay, western blotting, ELISA, etc.). Designing such assays is not always easy, especially if only the nucleotide sequence coding for the target protein has been obtained from a sequencing project and no known activity is associated with the predicted polypeptide.

Interacting protein partners may also be recovered by passing cellular extracts or cellular fractions through columns containing the target protein as a bait. A prerequisite for this strategy is the recovery of a substantial amount of (recombinant) bait protein to build the affinity column. This strategy will not be successful, however, if the endogenous bait proteins form tight complexes with interacting partners, preventing their retention on the column. Additional problems may be encountered if the bait protein needs to be modified posttranslationally for successful association, if interacting partners are present in minute amounts, and/or if abundant cellular proteins interact nonspecifically with the bait protein, masking the specific interacting partners present at low level.

Coimmunoprecipitation (see Chapter 5 and Sambrook and Russell 2001, *Molecular Cloning*, pp. 18.60–18.68) is a very useful method to copurify interacting protein partners. The success of this strategy depends on the availability of good-quality antibodies directed against the target protein. This often requires the production of recombinant target protein or a fragment thereof to generate monoclonal or polyclonal antibodies of high affinity and specificity. Indeed, immunopurification of target protein and interacting partners is most often performed in a single step. Thus, the efficiency of the method and the purity of the recovered material will be totally dependent on the quality of the antibodies used. In addition, because this procedure involves a single purification step, this limits the use of this strategy for protein present in very low abundance. Furthermore, the purified material is often contaminated by immunoglobulins used for the purification that may mask unknown protein partners. A strength of the coimmunoprecipitation strategy is, however, that proteins associated *in vivo* are copurified in a rapid and simple manner. Therefore, coimmunoprecipitation remains a rigorous method to validate the physiological significance of protein interaction.

Recently, we have developed a new strategy for protein purification that is particularly well suited for protein complex purification and for the identification of interacting protein partners. This strategy, dubbed TAP (for *tandem affinity purification*; Rigaut et al. 1999), involves the fusion of a protein tag to the target protein and its expression in a natural host cell or organism. Extracts prepared from these cells or organisms are then used to recover the target protein and associated interacting partners in a standard two-step process. The purified material can then be fractionated on a gel, and copurified proteins can be identified by mass spectrometry or Edman degradation. The TAP strategy combines several advantages of the standard biochemical purification and the coimmunoprecipitation strategies. As for classic biochemical purification, the successive purification steps of the TAP strategy allow the recovery of highly purified protein complexes present in low amounts in the starting extract. However, the use of a constant peptide tag and standard purification conditions avoids the problem of designing new purification schemes

for each new target that is associated with classic biochemical purification. Like coimmunoprecipitation, the TAP strategy relies on specific protein–protein (affinity matrix-tag) interaction of high affinity allowing the rapid, selective, and efficient recovery of the in-vivo-associated target complex from extracts. However, in contrast to coimmunoprecipitation, the various steps of the TAP method reduce background contamination by abundant cellular proteins or material leaking from the affinity column. This allows the recovery of highly purified complexes present at very low concentration. Like any method, the TAP strategy has some limitations (discussed below). Nevertheless, it appears applicable to a wide variety of proteins. Indeed, the TAP strategy has proven extremely useful in our hands and in other laboratories as a generic method to quickly purify protein complexes and associated proteins, overcoming a limiting step in the analysis of protein interaction by mass spectrometry.

## Tag Selection and Design

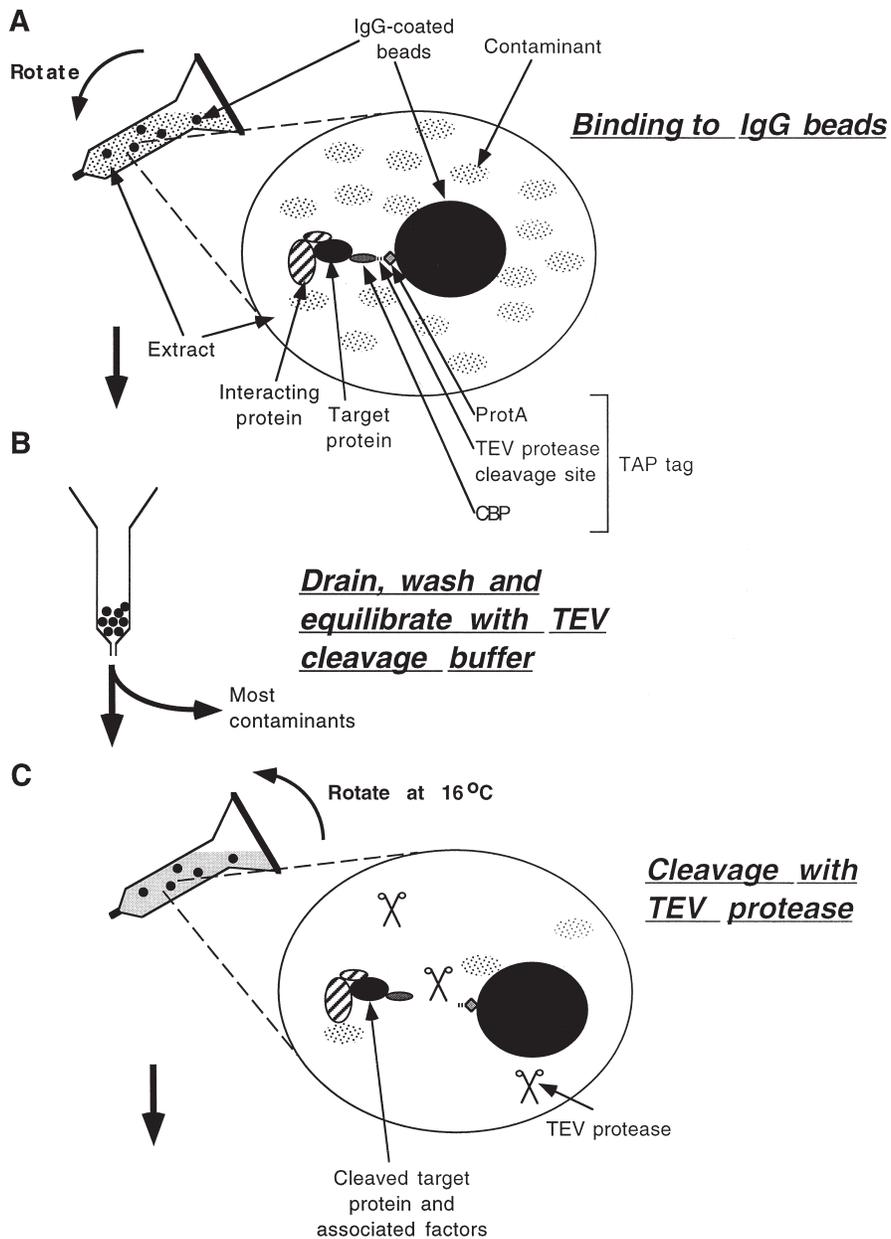
Optimal tags for protein complex purification and the analysis of protein interaction should have the following characteristics:

1. High affinity for the cognate matrix for quantitative recovery of low-abundance target proteins in dilute solutions.
2. Highly specific binding to increase the ratio of specifically to nonspecifically bound material to the affinity material.
3. Efficient and specific elution allowing high-level and specific recovery of the target protein.
4. Mild conditions of elution to preserve protein interactions and protein complex structure.

These characteristics are obviously somewhat contradictory, because efficient elution and/or elution under mild conditions is often difficult if a high-affinity interaction is involved. Similarly, efficient elution often requires harsh conditions that are not compatible with the preservation of protein–protein interaction. In addition, optimal tags should be as neutral as possible for the activity of the passenger protein and should work under a wide variety of salt and detergent conditions to allow purification.

To select an efficient tag for protein purification, we screened various available tags such as FLAG (Brizzard et al. 1994; Sambrook and Russell 2001, *Molecular Cloning*, pp. 15.4–15.6), His (Hochuli et al. 1988), Strep (Schmidt and Skerra 1993; Sambrook and Russell 2001, *Molecular Cloning*, pp. 11.118–11.119), two immunoglobulin G (IgG) binding units of *Staphylococcus aureus* Protein A (ProtA, Lohman et al. 1989), calmodulin-binding peptide (CBP, Stofko-Hahn et al. 1992), and chitin-binding domain (CBD, Chong et al. 1997) for optimal characteristics. Efficiency of recovery of a low-abundance passenger protein from yeast and tag selectivity (level of nonspecific background binding to the affinity support) was assayed semiquantitatively. Complementation analysis indicated that none of these tags impaired function of the Lsm3 target protein. In our hands, ProtA and CBP turned out to be the most efficient tags, with approximately 80% and 50% recovery of the target complex, respectively (Rigaut et al. 1999).

Binding of CBP to calmodulin in the presence of calcium is efficient and highly specific. Furthermore, this interaction can be easily reversed by the removal of calcium (by addition of EGTA; Stofko-Hahn et al. 1992). This represents a mild condition that does not appear to affect protein–protein interaction in many complexes. The binding of ProtA to rabbit IgG is also very efficient and highly specific (Lohman et al. 1989). However, elution of the bound material usually requires harsh conditions (e.g., very low pH) that would not preserve protein complex integrity and would also cause elution of material nonspecifically bound to the affinity matrix. Therefore, we decided to include a protease cleavage site between the passenger protein and the ProtA tag to allow specific and efficient elution under mild conditions. The TEV protease was selected for this purpose because of its high specificity and commercial availability (see Materials)



**FIGURE 1.** (Continues on facing page.) Theoretical and practical overview of the TAP method. The various steps of the TAP method are shown. Technical conditions are indicated for each step. When relevant, a detailed description of the reaction occurring at a specific step is provided in an enlarged view. The names of the various proteins, enzymes, and affinity media are indicated.

and because relatively mild conditions are required for efficient cleavage activity (Dougherty et al. 1989). Preliminary experiments demonstrated that single-step purifications were not sufficient to recover highly purified proteins from total cellular extracts. Therefore, we combined the CBP tag and ProtA flanked by a TEV protease cleavage site to create the TAP tag (Rigaut et al. 1999). Purification of a TAP-tagged protein present in a crude extract involves four main steps (Fig. 1):

1. Binding of the target protein to the IgG beads (Fig. 1A).

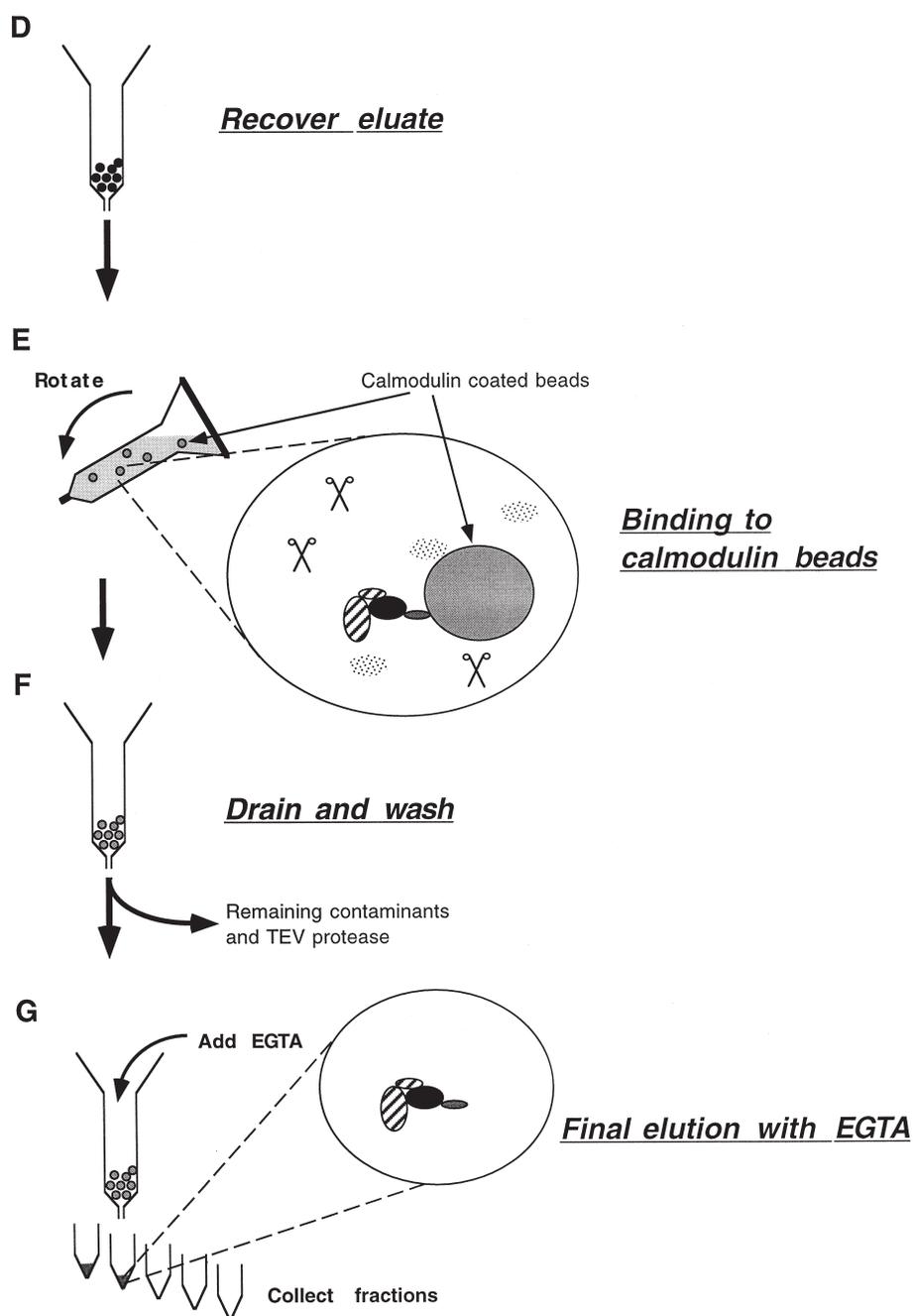


FIGURE 1. (See facing page for legend.)

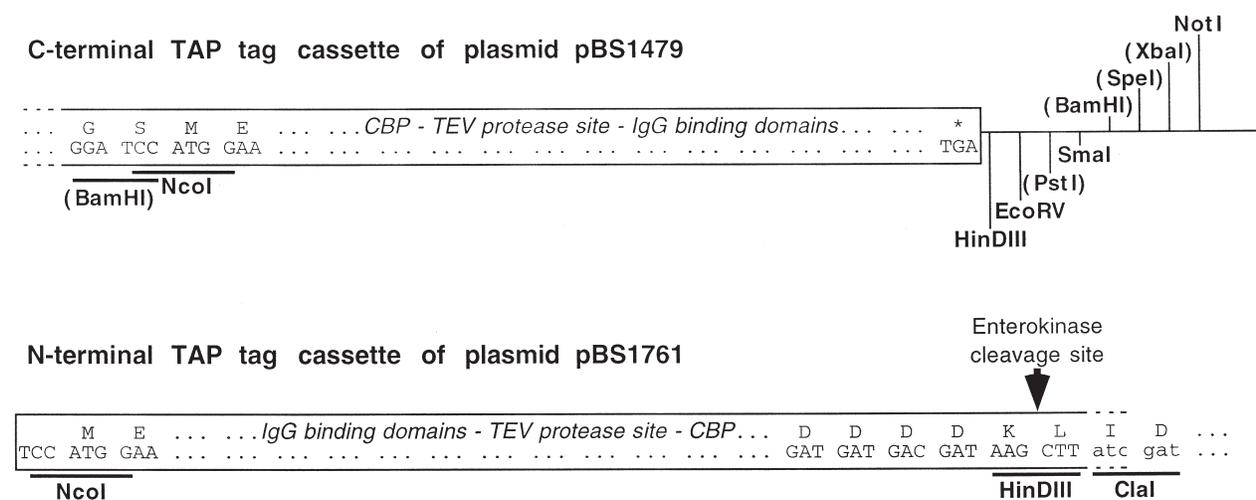
2. After extensive washing (Fig. 1B), elution of the bound material with the help of the TEV protease (Fig. 1C).
3. Binding of the protein present in the first eluate (Fig. 1D) to calmodulin-coated beads in the presence of calcium (Fig. 1E).
4. After extensive washing (Fig. 1F), elution of the bound material by addition of EGTA (Fig. 1G).

It is noteworthy that the four different binding and elution steps of this procedure are highly specific, thereby increasing the level of purification of the target protein complex. Although all of these steps are not necessary for abundant proteins, they are required when the target is present at a low level. Theoretically, binding to IgG or to calmodulin could be used indifferently as the first purification step. However, because elution from IgG requires the addition of the TEV protease that will contaminate the material eluted from this affinity material, it is preferable to perform affinity purification on an IgG matrix first. The second affinity step then allows the removal of contaminating TEV protease in addition to nonspecific contaminants. As a consequence, TEV protease cleavage should remove only the protein A (ProtA) part of the TAP tag, leaving the CBP fused to the target protein behind. ProtA should therefore preferably be located at the extreme amino or carboxyl terminus of the passenger protein. Due to this constraint, amino- and carboxy-terminal TAP tags containing the CBP, TEV protease cleavage site, and ProtA moiety of the TAP tag in reverse order have been constructed (Puig et al. 2001). Because of the modularity of the TAP tag, the TAP method is highly flexible. Indeed, on some occasions, one may prefer to use a “split TAP tag” by fusing the CBP and TEV-ProtA fragment cassettes to two different passenger proteins, allowing the purification of subcomplexes containing the two target proteins simultaneously (Caspary et al. 1999). Alternatively, it is possible in some cases to subtract undesired proteins by fusing them to a ProtA cassette lacking a TEV protease cleavage site (Bouveret et al. 2000).

Vectors encoding amino- and carboxy-terminal TAP tag cassettes have been built (Rigaut et al. 1999; Puig et al. 2001). The structures of these cassettes, including the location of useful restriction sites, are presented in Figure 2. It is noteworthy that, in the case of the amino-terminal TAP tag, an additional cleavage site for the enterokinase protease has been added, allowing the (near) complete removal of the TAP tag from the target protein following the second purification step.

## OUTLINE OF PROCEDURE

The procedure involves construction of cells or organisms expressing the TAP-tagged protein, preparation of extracts, purification of the target protein according to the TAP procedure, and analysis of the purified complex as originally reported by Rigaut et al. (1999).



**FIGURE 2.** Structures of the carboxy- and amino-terminal TAP tags. The tags are shown as open boxes, and nucleotides and amino acids relevant for in-frame fusion are shown. Useful restriction sites are also indicated. The enzymes in brackets are not unique in the corresponding vectors, but they may still be used for the fusion of the TAP tag to the target open reading frame.

## Construction of Cells or Organisms Expressing a TAP-tagged Protein

When constructing a protein fusion, one should also consider how the vector carrying the tagged fusion will be introduced in the recipient cell or organism and how expression of the fusion protein will be controlled. Because the goal of a TAP purification experiment is often to identify a physiological partner(s), one should express the tagged protein in cells or organisms naturally expressing the target protein or in the closest possible system available. This feature may become critically important in multicellular organisms because the target protein may interact with different partners in different tissues or at different developmental stages. It is also important to avoid high overexpression of the target protein. Although this strategy is often used to out-compete binding of the endogenous target protein with natural partners, exaggerated overexpression of the target protein most often results in the formation of a pool of free protein because natural interacting partners are not themselves overexpressed. Target proteins present in this free pool frequently interact with nonnatural partners such as heat-shock factors, the proteasome, or abundant cellular proteins (e.g., translation factors or glycolytic enzymes) (Swaffield et al. 1995, 1996). If the free pool of target protein is in large excess over interacting partners, it is possible that the nonnatural partners will be in excess or even mask natural interacting partners in the purified fraction. Optimally, one should therefore replace the gene(s) coding for the target protein by a TAP-tagged version. However, this is not always possible. Nevertheless, these considerations should be taken into account in building the protein fusion.

Usually, a standard cloning technique can be used for constructing the appropriate vector carrying the TAP tag fused to the target protein (see Sambrook and Russell 2001, *Molecular Cloning*, Chapters 1–4 for vector cloning methods). This vector is then introduced into recipient cells or organisms following the best procedure available for this specific system. However, in some cases, strategies bypassing the cloning step are available. A striking example is provided by the yeast *Saccharomyces cerevisiae*, where the endogenous target gene can be replaced by a TAP-tagged version through the simple transformation of a wild-type strain with a PCR fragment (Baudin et al. 1993; Puig et al. 1998).

## Preparation of Extracts

Even though progress in mass spectrometry has significantly increased the sensitivity of protein identification methods, a significant amount of cells will be required for protein purification. Using the TAP method, this quantity can be determined empirically by comparing, using western blotting, the level of expression of the target protein to the level of expression of a previously purified protein carrying the TAP tag (e.g., by using either the Mud13p, Snu71p, or Lsm9p protein [Rigaut et al. 1999] as control). Extracts can be prepared using a variety of conditions. They can be prepared from cell culture, tissues (from transgenic organisms), or even whole organisms. Obviously, any step increasing the number of cells expressing the target protein relative to other proteins will facilitate the purification. This could involve the use of tissues or homogeneous cell cultures instead of whole organisms or the use of cellular fractions instead of whole cellular extracts. Extracts should be prepared in conditions that optimize target protein yield and solubility without interfering with protein–protein interaction. In some cases, these conditions may be nearly impossible to satisfy, because interaction of the target protein with some structure may be more difficult to break up than interaction of the target protein with another protein. (For example, solubilization of a membrane protein may require conditions that destroy interaction of the target protein with specific interacting partners.)

To establish a basis for the design of the extract preparation procedure, it is usually useful to explore the literature to define conditions that have been used for preparation of extracts using the same cell system or for purifying related complexes. Pilot experiments can be used to determine optimal conditions for protein extraction. However, one should remember that stronger

conditions might increase the target protein yield while concomitantly reducing the level of the interacting partners. It is also possible that the target protein will interact with various partners and that these various complexes will be extracted with very different efficiencies. Generally, increasing salt and/or detergent concentrations increases protein release but tends to disrupt natural protein interactions, whereas low salt and detergent concentrations reduce protein release while favoring nonspecific interactions. If possible, we suggest using conditions that mimic the intracellular environment of the target protein and that should therefore preserve its solubility and interaction potential. One should remember, however, that any change in conditions during extract preparation may irreversibly disrupt a protein interaction: Even if proper conditions are restored afterward, the interacting partner may be unfolded, modified, or present at too low a concentration to allow reassociation.

It is also noteworthy that, during extract preparation, components of different cellular organelles or of different cell types are being mixed together, creating opportunities for proteins to form nonnatural associations. This situation also favors protein degradation and denaturation. This should be prevented as much as possible by reducing incubation time, lowering the temperature, and/or including protease inhibitors. Therefore, optimal conditions for protein extraction will vary from protein to protein and from one cell type to another. They should be determined empirically following the guidelines given above. The possibility of detecting a small amount of the TAP-tagged target protein by western blotting provides a useful tool for testing various extraction conditions at reduced scale in pilot experiments. Extracts may be used directly for purification or may be stored frozen after addition of glycerol or after dialysis against a glycerol-containing buffer. In some cases, it may be helpful to remove insoluble material from the extract by centrifugation before using it for purification. (An example of extract preparation conditions is given for the yeast *Lsm3* protein on p. 322.)

## Tandem Affinity Purification

The various steps of the TAP procedure are depicted in Figure 1. The first affinity selection is performed in a batch format by mixing the extract with IgG beads (Fig. 1A). Unbound material is removed by emptying the column by gravity, and nonspecifically bound material is removed by extensive washing with the binding buffer (Fig. 1B). Beads are then washed with TEV cleavage buffer (see Materials, pp. 324–325) before performing the TEV cleavage reaction (Fig. 1C). After cleavage, the eluate (containing the TEV protease) is recovered (Fig. 1D), diluted in the appropriate buffer, and incubated in batch with calmodulin-coated beads (Fig. 1E). Contaminants and the TEV protease are removed by extensive washing (Fig. 1F) before elution of the purified complex with EGTA (Fig. 1G). From some test experiments, we have estimated the overall yield of the method to be roughly 20–30% recovery of the starting material. The purified material can be kept frozen before analysis. However, if functional tests are planned, the purified material may have to be stored in a buffer containing glycerol or another component to preserve its activity. This may be achieved through dialysis or direct addition of requested chemicals (e.g., glycerol) to the eluate. In this vein, it is noteworthy that even if the last elution step of the TAP procedure disrupts the target complex (e.g., if stability of this complex requires calcium), this will not affect the analysis of the composition of the complex, although this is likely to disrupt its activity.

The various reagents used for the TAP purification are described in the Materials section (p. 324), and a detailed step-by-step protocol is given in the Methods section. As for extract preparation, conditions may be adapted for different target proteins (salt concentration, detergents, etc.; see above). Similarly, reducing the temperature and the handling time as well as including protease inhibitors may help to preserve the integrity of proteins and protein complexes. Extensive washing steps should be included between the two binding and elution steps to prevent contamination of the purified fraction with background material. Similarly, important changes in buffer

conditions should be avoided because they tend to provoke the release of material bound non-specifically to the affinity matrix and because they could affect the stability of the target protein complex. A potential concern with the TAP method is that calmodulin from the host cell might interact with the CBP moiety of the TAP tag and prevent its association with the affinity matrix during the second step of the purification. Although this is theoretically possible, we have not yet encountered such a situation. Yet, if this becomes a problem, inclusion of EGTA during extract preparation and/or the first purification step on IgG-coated beads should remove endogenous calmodulin, exposing CBP for the second affinity step.

## Analysis of the Purified Material

Material recovered from a TAP purification may be analyzed in various ways, including testing its activity *in vitro*, analyzing the structural shape of the complex by electron microscopy, and assaying for the presence of candidate protein(s) by western blotting (Rigaut et al. 1999; O. Puig et al., unpubl.). In the analysis of interacting proteins, mass spectrometry appears to be the method of choice for protein identification. We routinely fractionate the purified material on monodimensional denaturing protein gels. Gels are poured with an exponential gradient of acrylamide concentration to favor resolution of proteins in a wide variety of molecular-weight ranges. Great care should be taken in handling such gels to avoid introduction of human keratin that could compromise analysis of the purified products by mass spectrometry. Gels are stained with Coomassie Blue or with silver nitrate. A scan of the gel is performed before cutting out the desired band for analysis by mass spectrometry. An advantage of protein fractionation before analysis by mass spectrometry is that one can get a rough estimate of the complex abundance, the number of proteins present, and their relative stoichiometric levels. However, it is possible that some material is lost during this process and that direct analysis of the eluted material by mass spectrometry (Link et al. 1999) could be more sensitive.

## Controls and Verification

To control for the specificity of the copurification, one can perform a purification with extracts prepared from cells that do not express a TAP-tagged protein. Although this is an important control, the mere fact that a protein is recovered in the purified fraction with a given TAP-tagged protein but is not recovered in a purification with material obtained from a nontagged strain does not demonstrate that this interaction is specific. Indeed, interaction with the target protein may represent interaction between partly denatured proteins or the formation of aggregates. The comparison of several purifications using unrelated target proteins is often instructive. Indeed, if the same proteins are repeatedly recovered in the purified fractions, they are likely to represent contaminants, particularly if they represent abundant cellular proteins (e.g., translation factors) or proteins known to interact with unfolded polypeptides (e.g., heat shock proteins, proteasome). In any case, it is important to verify that a protein identified in a TAP-purified fraction interacts physiologically with the target protein using an independent method. Coimmunoprecipitation (see Chapter 5) appears as a method of choice for such verification. In the absence of antibody, the ProtA and CBP tags can be used to test such coprecipitations. First, one of the target proteins is tagged with CBP (or a full TAP tag) while the putative interacting partner is tagged with ProtA (or the fusion TEV protease cleavage site-ProtA). Cells expressing both fusion proteins are lysed and incubated with calmodulin-coated beads in the presence of calcium. Samples from the input extracts, supernatant fraction, and purified material are then fractionated by gel electrophoresis before detecting the ProtA-protein fusion(s) by western blotting (Salgado-Garrido et al. 1999; Bouveret et al. 2000).

## Protocol 1

# Preparation of Extracts to Purify the Yeast Lsm3 Protein

Using this protocol, more than 20 proteins ranging from 8 to 250 kD were visible by Coomassie staining in the purified fraction and were identified by mass spectrometry (Bouveret et al. 2000). Quantification of the associated U6 snRNA by primer extension indicated that about 25–40% of the starting extract material was recovered in the final fraction. The Lsm3 protein belongs to the Sm protein family (Séraphin 1995) and is used here as a representative sample.

## MATERIALS

---

**CAUTION:** See Appendix for appropriate handling of materials marked with <!.>.

### Buffers and Solutions

#### Buffer A

- 10 mM K-HEPES (pH 7)
- 10 mM potassium chloride (KCl) <!.>
- 1.5 mM magnesium chloride (MgCl<sub>2</sub>) <!.>
- 0.5 mM dithiothreitol (DTT) <!.>
- 0.5 mM phenylmethylsulfonyl fluoride (PMSF) <!.>
- 2 mM benzamidine
- 0.5 µg/ml (1 µM) leupeptin <!.>
- 1.4 µg/ml (2 µM) pepstatin A <!.>
- 2.4 µg/ml (4 µM) cymostatin
- 17 µg/ml (2.6 µM) aprotinin <!.>

#### Buffer D

- 20 mM K-HEPES (pH 7.9)
- 50 mM KCl <!.>
- 0.2 mM EDTA (pH 8.0)
- 0.5 mM DTT <!.>
- 20% glycerol
- 0.5 mM PMSF <!.>
- 2 mM benzamidine

### Additional Reagents

- LSM3*
- Tag for protein purification

### Special Equipment

- French press

## METHOD

---

1. To identify interacting factors in yeast (or another protein of interest), fuse a cassette encoding the TAP tag to the carboxyl terminus of the *LSM3* reading frame using standard cloning techniques on a low-copy centromeric plasmid. The resulting construct should complement a disruption of the essential *LSM3* gene to indicate that it is fully functional.
2. Grow up 2 liters of culture of the resulting strain to saturation.
3. Recover cells by centrifugation, wash with H<sub>2</sub>O, pellet again, and freeze at -80°C before extract preparation.
4. Resuspend the cells in one pellet volume of Buffer A. Break by passing two times through a French press at 8.27 MPa (1200 psi) at 4°C.
5. Adjust this crude extract to 200 mM KCl using a 2 M stock.
6. Remove cell debris by two consecutive centrifugation steps at 4°C (50,000g for 30 minutes followed by 130,000g for 85 minutes).
7. Dialyze the cleared extracts for 3 hours against Buffer D at 4°C and store frozen at -80°C before using for TAP purification using the protocol described below. The final volume of extract from 2 liters of starting culture should be approximately 10 ml.

## Protocol 2

# Dissecting Protein Interactions with the TAP Method

The TAP method can be used to identify interacting partners, and it can also be used to characterize protein interactions occurring in a complex in more detail. For example, purification of a mutant or a truncated protein fused to the TAP tag can be used to map interaction domains. Similarly, complexes may be purified from cells carrying a mutation or even a deletion of one of the complex subunits that is different from the target protein. This type of analysis can give some insight into interactions occurring in the complex or on the assembly pathway of this complex. Repeating the TAP purification of a protein complex under various conditions (e.g., increased salt or detergent concentration) may also be used to differentiate protein(s) interacting directly with the target protein from more peripheral subunits. Phylogenetic comparison of complexes may also be instructive because different subcomplexes may exist in various species, giving information about protein interaction order. Furthermore, phylogeny may be used to identify protein partners. For example, if it is not possible to purify a protein from a given species, one may consider purifying its homolog from a species where the TAP method is easy to implement. This strategy has proven useful in the analysis of an RNA helicase involved in mRNA transport (Schmitt et al. 1999).

## MATERIALS

---

**CAUTION:** See Appendix for appropriate handling of materials marked with <!.>.

### Buffers and Solutions

Unless otherwise stated, all buffers are cooled to the working temperature of 4°C before use.

#### IPP150 buffer

- 10 mM Tris-Cl (pH 8.0)
- 150 mM NaCl
- 0.1% Nonidet P-40 (NP-40)

#### IPP150 calmodulin-binding buffer

- 10 mM  $\beta$ -mercaptoethanol (dilute and add immediately before use) <!.>
- 10 mM Tris-Cl (pH 8.0)
- 150 mM NaCl
- 1 mM magnesium acetate <!.>
- 1 mM imidazole <!.>
- 2 mM  $\text{CaCl}_2$
- 0.1% NP-40

#### IPP150 calmodulin elution buffer

- 10 mM  $\beta$ -mercaptoethanol (dilute immediately before use) <!.>
- 10 mM Tris-Cl (pH 8.0)
- 150 mM NaCl
- 1 mM magnesium acetate <!.>
- 1 mM imidazole <!.>
- 2 mM EGTA
- 0.1% NP-40

## Enzymes and Buffers

TEV cleavage buffer  
10 mM Tris-Cl (pH 8.0)  
150 mM NaCl  
0.1% NP-40  
0.5 mM EDTA  
1 mM DTT (add immediately before use from 1 M stock) <!\>  
TEV protease (GIBCO 10127-017)

## Affinity Media

Calmodulin affinity resin (Stratagene 214303)  
IgG agarose (Sigma A 2909) or IgG Sepharose (Pharmacia 17-0969-01)

## Extracts

Appropriate cell lines or strains expressing a TAP-tagged protein to prepare extracts. As a guideline, we store yeast extracts before purification in 20 mM HEPES (pH 7.9) (adjusted with KOH), 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT <!\>, 20% glycerol, 0.5 mM PMSF <!\>, 2 mM benzamidine.

## Special Equipment

Cold room space  
Incubator (16°C) with rotating wheel  
Poly-Prep Chromatography Column (10-ml; Bio-Rad 731-1550)  
Rotating wheel

## METHOD

---

1. Wash 200  $\mu$ l of IgG agarose or IgG Sepharose beads with 5 ml of IPP150 buffer in a closed purification column. Rotate the beads and wash buffer in the cold room for 5 minutes. Remove the IPP150 buffer by letting the column drain by gravity.
2. Adjust the extract buffer concentration by adding 50  $\mu$ l of 2 M Tris-Cl (pH 8.0), 200  $\mu$ l of 5 M NaCl, and 100  $\mu$ l of 10% NP-40 per 10 ml of extract.
3. Add equilibrated extract (10 ml) to the washed beads. Close the column (top and bottom). Rotate for 2 hours at 4°C.
4. Remove the unbound material by opening the column (top first) and letting it drain by gravity. This fraction may be saved for control analysis (e.g., estimation of the binding efficiency by western blotting).
5. Wash the column with 3  $\times$  10 ml of IPP150 buffer.  
Careful cleaning of the column wall and column can help in reducing the level of contaminants.
6. Equilibrate the column for the TEV cleavage reaction by washing the column with 10 ml of TEV cleavage buffer at 16°C.

7. Close the bottom of the column. Add 1 ml of TEV cleavage buffer and 100 units of TEV protease. Close the top of the column. Rotate for 2 hours at 16°C.
8. Remove first the top and then the bottom plug of the column. Recover the eluate by gravity flow. The solution remaining in the column dead-volume and on the column walls may be eluted with an additional 200  $\mu$ l of TEV cleavage buffer.

Material remaining on the column may be eluted with a buffer containing SDS, such as gel loading buffer or buffer 1x PK (100 mM Tris-Cl, pH 7.5, 12.5 mM EDTA, pH 8.0, 150 mM NaCl, 1% SDS) and saved for control analysis.
9. Wash 200  $\mu$ l of calmodulin affinity resin with 5 ml of IPP150 calmodulin-binding buffer in a new purification column. Rotate the beads and the IPP150 calmodulin-binding buffer in the closed column for 5 minutes in the cold room. Remove the IPP150 calmodulin-binding buffer by letting the column drain by gravity.
10. To the previous eluate add 3 volumes of IPP150 calmodulin-binding buffer and 1  $\mu$ l of 1 M  $\text{CaCl}_2$  per milliliter of eluate to titrate the EDTA present in the TEV cleavage buffer.
11. Transfer the equilibrated solution to the column containing the washed calmodulin affinity resin. Close the column (top and bottom). Rotate for 2 hours at 4°C.
12. Remove the unbound material by opening the column (top first) and letting it drain by gravity. This fraction may be saved for control analysis.
13. Wash the column with 3  $\times$  10 ml of IPP150 calmodulin-binding buffer.

Careful cleaning of the column wall may reduce the level of contaminants.
14. Elute 5 fractions of 200  $\mu$ l with IPP150 calmodulin elution buffer.

Proteins present in these fractions may be dialyzed against the appropriate buffer for functional analysis or concentrated (e.g., by TCA or methanol-chloroform precipitation; Wessel and Flugge 1984; Ozols 1990) before fractionation on a denaturing gel.

## TROUBLESHOOTING AND LIMITATIONS

---

It is often valuable to follow quantitatively the purification of the target protein by western blotting. This allows the determination of problematic steps and the design of potential solutions. It is also valuable to obtain cells expressing a known TAP-tagged fusion protein as a positive control for the first purification to check that the various buffers and affinity material are fully functional.

Although the TAP method is broadly applicable, there are some limitations. First, a functional TAP-tagged protein must be produced. For some proteins, tagging at both the amino and the carboxyl termini of the protein may affect its activity. In our experience, however, this is uncommon. Even if the protein is functional, a similar problem may occur if the TAP tag is inaccessible in the native protein complex. In this case, we suggest testing a construct expressing the target protein fused to the TAP tag at its other extremity. Although some proteins also contain an endogenous TEV protease cleavage site that interferes with the purification, this is not likely to be a very common problem. Indeed, as an example, only a dozen polypeptides among the 6000 yeast proteins are predicted to contain a TEV protease cleavage site (consensus sequence ENLYFQG, Dougherty et al. 1989). One should remember, however, that the TEV protease is not a restriction enzyme and that degenerate sites may be cleaved if well exposed, whereas perfect sites buried in the interior of the protein will not be accessible to the protease. The presence of a bona fide TEV protease cleavage site in the target protein remains to be determined by an experimental approach.

## REFERENCES

- Baudin A., Ozier K.O., Denouel A., Lacroute F., and Cullin C. 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **21**: 3329–3330.
- Bouveret E., Rigaut G., Shevchenko A., Wilm M., and Séraphin B. 2000. A Sm-like protein complex that participates in mRNA degradation. *EMBO J.* **19**: 1661–1671.
- Brizzard B.L., Chubet R.G., and Vizard D.L. 1994. Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *BioTechniques* **16**: 730–735.
- Caspary F., Shevchenko A., Wilm M., and Séraphin B. 1999. Partial purification of the yeast U2 snRNP reveals a novel yeast pre-mRNA splicing factor required for pre-spliceosome assembly. *EMBO J.* **18**: 3463–3474.
- Chong S., Mersha F.B., Comb D.G., Scott M.E., Landry D., Vence L.M., Perler F.B., Benner J., Kucera R.B., Hirvonen C.A., Pelletier J.J., Paulus H., and Xu M.Q. 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **192**: 271–281.
- Deutscher M.P. 1990. *Guide to protein purification*. Academic Press, San Diego.
- Dougherty W.G., Cary S.M., and Parks T.D. 1989. Molecular genetic analysis of a plant virus polyprotein cleavage site: A model. *Virology* **171**: 356–364.
- Gygi S.P., Corthals G.L., Zhang Y., Rochon Y., and Aebersold R. 2000. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci.* **97**: 9390–9395.
- Hochuli E., Bannwarth W., Doebeli H., Gentz R., and Stueber D. 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adjustment. *Bio/Technology* **6**: 1321–1325.
- Lamond A.I. and Mann M. 1997. Cell biology and the genome projects—A concerted strategy for characterizing multi-protein complexes using mass-spectrometry. *Trends Cell Biol.* **7**: 139–142.
- Link A.J., Eng J., Schieltz D.M., Carmack E., Mize G.J., Morris D.R., Garvik B.M., and Yates J.R., III. 1999. Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **17**: 676–682.
- Lohman T.M., Chao K., Green J.M., Sage S., and Runyon G.T. 1989. Large-scale purification and characterization of the *Escherichia coli* rep gene product. *J. Biol. Chem.* **264**: 10139–10147.
- Ozols J. 1990. Amino acid analysis. *Methods Enzymol.* **182**: 587–601.
- Puig O., Rutz B., Luukkonen B.G., Kandels L.S., Bragado N.E., and Séraphin B. 1998. New constructs and strategies for efficient PCR-based gene manipulations in yeast. *Yeast* **14**: 1139–1146.
- Puig O., Caspary F., Rigaut G., Rutz B., Bouveret E., Bragado-Nilsson E., Wilm M., and Séraphin B. 2001. The Tandem Affinity Purification (TAP) method: A general procedure of protein complex purification. *Methods* **24**: 218–229.
- Rigaut G., Shevchenko A., Rutz B., Wilm M., Mann M., and Séraphin B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**: 1030–1032.
- Salgado-Garrido J., Bragado-Nilsson E., Kandels-Lewis S., and Séraphin B. 1999. Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. *EMBO J.* **18**: 3451–3462.
- Sambrook J. and Russell D.W. 2001. *Molecular cloning: A laboratory manual*, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schmidt T.G. and Skerra A. 1993. The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. *Protein Eng.* **6**: 109–122.
- Schmitt C., von Kobbe C., Bachi A., Pante N., Rodrigues J.P., Boscheron C., Rigaut G., Wilm M., Séraphin B., Carmo-Fonseca M., and Izaurralde E. 1999. Dbp5, a DEAD-box protein required for mRNA export, is recruited to the cytoplasmic fibrils of nuclear pore complex via a conserved interaction with CAN/Nup159p. *EMBO J.* **18**: 4332–4347.
- Séraphin B. 1995. Sm and Sm-like proteins belong to a large family: Identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *EMBO J.* **14**: 2089–2098.
- Shevchenko A., Jensen O.N., Podtelejnikov A.V., Sagliocco F., Wilm M., Vorm O., Mortensen P., Shevchenko A., Boucherie H., and Mann M. 1996. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci.* **93**: 14440–14445.
- Stofko-Hahn R.E., Carr D.W., and Scott J.D. 1992. A single step purification for recombinant proteins. Characterization of a microtubule associated protein (MAP 2) fragment which associates with the type II cAMP-dependent protein kinase. *FEBS Lett.* **302**: 274–278.
- Swaffield J.C., Melcher K., and Johnston S.A. 1995. A highly conserved ATPase protein as a mediator between acidic activation domains and the TATA-binding protein. *Nature* **374**: 88–91.
- . 1996. A highly conserved ATPase protein as a mediator between acidic activation domains and the

- TATA-binding protein (Correction). *Nature* **379**: 658.
- Wasinger V.C., Cordwell S.J., Cerpa-Poljak A., Yan J.X., Gooley A.A., Wilkins M.R., Duncan M.W., Harris R., Williams K.L., and Humphery-Smith I. 1995. Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. *Electrophoresis* **16**: 1090–1094.
- Wessel D. and Flugge U.I. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**: 141–143.
- Wilkins M.R., Pasquali C., Appel R.D., Ou K., Golaz O., Sanchez J.C., Yan J.X., Gooley A.A., Hughes G., Humphery-Smith I., Williams K.L., and Hochstrasser D.F. 1996. From proteins to proteomes: Large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Bio/Technology* **14**: 61–65.
- Wilm M., Shevchenko A., Houthaeve T., Breit S., Schweigerer L., Fotsis T., and Mann M. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**: 466–469.