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Identification of SUMO-Interacting Proteins by Yeast Two-Hybrid Analysis

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i. Summary

This chapter will discuss various adaptations of the yeast two-hybrid method for analyzing protein interactions that can be used to identify small ubiquitin-related modifier (SUMO)-interacting proteins and to determine the nature of the SUMO-protein interactions that occur. SUMO binds to a protein in two different ways, covalently and noncovalently. In a covalent interaction an isopeptide bond forms between the glycine residue at the C-terminus of mature SUMO and a lysine side-chain on the substrate protein. Alternatively, SUMO can interact noncovalently with another protein, usually via insertion of a β strand from a substrate SUMO-interacting motif (SIM) into a hydrophobic groove next to the SUMO β 2 strand. By mutating either the C-terminal diglycine motif or amino acids within the β 2 strand of SUMO, these respective interactions can be abolished. Expression of two-hybrid SUMO constructs with either of these mutations can help distinguish the type of interaction that occurs between SUMO and a given protein. Sumoylation can be verified by independent methods, such as a SUMO mobility shift assay. Finally, the chapter will compare the two-hybrid approach with mass spectrometric analysis as a way to identify SUMO-interacting proteins.

Keywords

SUMO; two-hybrid analysis; SIM (SUMO-interacting motif); desumoylating enzymes/SUMO protease

1. Introduction

Mammalian SUMO-1 (small ubiquitin-related modifier-1) was initially identified as an interacting protein with various bait proteins in different yeast two-hybrid screens (1–3). Two independent studies then revealed that SUMO-1 acts as a modifying group that is covalently bound to a substrate protein (4,5). “Sumoylation” of a protein can change its localization, activity, or interaction with other proteins, and SUMO (there are three functional SUMO paralogs in mammals, SUMO-1 and the closely related SUMO-2 and SUMO-3 proteins; here we will use “SUMO” unless the paralogs need to be distinguished) has been implicated in a number of biological pathways including cell cycle progression, DNA repair, cytokinesis, and transcription (6,7). Therefore, a more comprehensive understanding of the proteins that interact with SUMO can shed light on the mechanisms by which SUMO regulates specific cellular regulatory mechanisms. Subsequent to the discovery of SUMO as a post-translational modifier, the yeast two-hybrid method – designed to identify *in vivo* protein-protein interactions (8) – has been utilized to identify a number of other proteins that interact with SUMO (9,10). One

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of the great advantages of this technique is that it not only identifies SUMO-interacting proteins, but it can be adapted so that covalent and noncovalent interactions with SUMO can be distinguished (9). This chapter will detail how the two-hybrid approach can be used to identify proteins that interact with SUMO and to distinguish whether the association with SUMO is likely to be covalent or noncovalent. We will also compare the two-hybrid approach to mass spectrometry methods as alternatives for the identification of SUMO-protein interactions.

2. Materials

1. Yeast two-hybrid vectors and expression strain, for example: pGAD and pGBD; pGAD-based plasmid library of yeast chromosomal DNA fragments, and yeast two-hybrid expression strain PJ69-4A (11)
2. Restriction enzymes, T4 DNA ligase
3. SD minimal medium plates lacking various combinations of the following: leucine, uracil, histidine, and adenine; and buffered SD minimal medium plates for yeast containing X-gal, 5-Fluoroorotic acid (5-FOA), or 3-amino-triazole (3-AT)
4. E.Z.N.A.TM Yeast Plasmid Mini Kit (Omega Bio-Tek, Inc.) or an equivalent system for harvesting plasmids from yeast
5. *E. coli* strain carrying a *leuB* mutation such as the RR1 strain (11) and M9 minimal medium plates lacking leucine
6. Oligonucleotide primers for DNA sequencing
7. Quikchange mutagenesis kit (Stratagene) or an equivalent system
8. *ULP1* over-expression plasmid (12)
9. TAP-tagged yeast strains (Open Biosystems)
10. Yeast high-copy SUMO plasmids, expressing either N-terminally tagged SUMO and untagged SUMO (9)
11. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reagents, equipment for protein electroblotting, and anti-PAP antibody (Sigma)

3. Methods

The methods described below outline: (1) the construction of the necessary yeast two-hybrid plasmids and the protocol for screening a “prey” library for SUMO-interacting proteins; (2) the variations used to determine if a SUMO interaction is likely to be covalent, noncovalent, or both; (3) a method used to verify that the identified protein is indeed sumoylated; and (4) a brief comparison of two-hybrid and mass spectrometry approaches.

3.1. Screening a yeast genomic library for two-hybrid interactions with SUMO

The two-hybrid screening methods used to identify SUMO-interacting proteins will be described in **Subheadings 3.1.1. –3.1.3.** These include a description of the plasmids and yeast strains used to screen a library of two-hybrid “prey” constructs for their interactions with the “bait” SUMO, as well as methods used to confirm the two-hybrid signal and to identify the interacting peptides or protein domains.

3.1.1. Two-hybrid plasmids and yeast strains—A number of vector systems and strains are available for yeast two-hybrid analysis. The analysis of SUMO-protein interactions

described here uses a set of constructs and yeast strains described previously (11). Figure 1 depicts the plasmids used for screening, the pGBD-SUMO bait plasmid and pGAD vector into which the target DNA library is inserted. The two-hybrid method exploits the ability of the DNA-binding and activation domains of a transcriptional activator to function on separate polypeptides if those polypeptides are able to associate. In the present case, the yeast Gal4 transcription factor is used. The Gal4 DNA-binding domain (GBD) and the Gal4 activation domain (GAD) must be brought into close proximity to activate reporter genes that are under the control of distinct *GAL* promoters. These Gal4 domains do not have an affinity for one another but are brought together by the interaction of the proteins to which they are each fused. The yeast two-hybrid reporter strain PJ69-4A was developed with three separate reporter genes: *GAL1-HIS3*, *GAL2-ADE2*, and *GAL7-lacZ*. The use of distinct *GAL* promoters and reporters helps to minimize false positives. The different reporters vary some in their sensitivity to particular protein-protein interactions. In general, the *GAL1-HIS3* is sensitive to even weak interactions, and the stringency of the growth assay can be increased by adding 3-amino-triazole (3-AT), an inhibitor of His3 catalytic activity, to the medium.

The SUMO-encoding bait gene (yeast *SMT3* in the current example) is cloned into the plasmid containing the DNA-binding domain of Gal4, yielding pGBD-SUMO. In the example study, a *Saccharomyces cerevisiae* genomic library derived from various partial restrict digests of chromosomal DNA was screened (11). Alternatively, cDNA libraries from yeast or other species could be utilized. Three versions of the pGAD plasmids (pGAD-C1, pGAD-C2, and pGAD-C3) allow for in-frame fusion of the Gal4 activation domain and various prey fragments (see Note 1). The pGBD-SUMO plasmid is first transformed into the PJ69-4A yeast cells to create the bait strain, and subsequently, the prey libraries are transformed into this strain. If the prey interacts with SUMO, the various reporter genes will yield a positive signal.

3.1.2. Screening a library for two-hybrid interactions with SUMO

1. Clone the SUMO-encoding gene into the multiple cloning site of the pGBD plasmid to express an in-frame full-length GBD-SUMO fusion. SUMO is fused to the C-terminal end of the GBD, so the SUMO C-terminus can be activated and conjugated to other proteins. SUMO proteins are normally expressed in precursor forms that require processing of C-terminal peptides in order to reach the mature, conjugation-competent state of the protein. To avoid problems with precursor processing, it is advisable to express only the mature portion of SUMO, which terminates with two glycine residues.
2. Obtain or create a library of yeast chromosomal DNA fragments cloned into each reading frame-specific pGAD plasmid, pGAD-C1, pGAD-C2, and pGAD-C3, or an analogous set of two-hybrid screening vectors.
3. Transform the library of pGAD plasmids into the two-hybrid yeast strain PJ69-4A carrying the pGBD-SUMO plasmid using standard techniques (13) (For an alternate approach using mammalian SUMO see Note 2). Cells should be plated at a density that will yield ~300–800 colonies per plate. This needs to be determined empirically.

¹Using random genomic fragments rather than full-length cDNAs in the construction of two-hybrid prey libraries can help narrow the specific region(s) of a protein that interacts with SUMO. Also there may be additional domains of a full-length prey fusion construct that impair the two-hybrid interaction between SUMO and the interacting region of the protein. Therefore, protein fragments may sometimes enhance detection of certain interactions by the yeast two-hybrid method.

²The SUMO isoforms (SUMO-1, SUMO-2, and SUMO-3) have been shown to sumoylate partially overlapping sets of substrates. A yeast two-hybrid screen comparing SUMO-1 and SUMO-2 has shown that these isoforms have distinct interactions (10). Future screens comparing the various SUMO isoforms might identify interesting subgroups of SUMO-interacting proteins.

4. Grow the cells on double dropout plates (SD–ura–leu, which are minimal medium plates lacking uracil and leucine) to select for cells that carry both two-hybrid plasmids.
5. To test for two-hybrid interactions, replica-plate colonies from the double dropout plates onto three different tester plates: (1) SD–ura–leu–his triple dropout plates (his = histidine), which test for the expression of the *GAL1-HIS3* reporter gene; (2) SD–ura–leu–ade triple dropout plates (ade = adenine), which test for the expression of the *GAL2-ADE2* reporter; and (3) X-gal plates lacking uracil and leucine, which test expression of the *GAL7-lacZ* reporter (colonies turn blue if the *lacZ*-encoded β -galactosidase enzyme is expressed).

Control transformations with empty pGAD vectors are also necessary to verify that any positive signal on the above plates requires the inserted prey sequence. If a high background of growth of cells expressing only GBD-SUMO is observed on plates lacking histidine, plates with different concentrations of 3-AT should be tested to find a minimal 3-AT concentration that prevents this growth. Ideally, a clone should give a positive signal on all three tester plates; however, weaker interactions might not always allow growth on the adenine dropout plates yet might still prove to be biologically relevant in subsequent validation tests.

3.1.3. Confirming the two-hybrid interaction and identifying the prey peptides—

Colonies yielding positive yeast two-hybrid signals express proteins that potentially interact with SUMO.

1. To confirm that the two-hybrid signal is dependent upon the SUMO fusion protein, evict the pGBD-SUMO plasmid from the yeast cells by streaking the colonies from the master plates onto medium containing 5-FOA. 5-FOA is toxic for strains that synthesize uracil, and therefore medium containing 5-FOA selects for the cells that have lost the *URA3*-marked pGBD-SUMO plasmid. 5-FOA-resistant cells are then retested for expression of the three two-hybrid reporters. If the two-hybrid signal is abrogated upon eviction of the pGBD-SUMO plasmid, the signal likely depends on the two-hybrid SUMO-prey protein interaction.
2. A second control to verify that a positive two-hybrid signal is due to interaction between the two fusion proteins is to recover the prey plasmid in *E. coli*, purify the plasmid, transform it into the original bait strain, and retest:
 - i. Isolate plasmid DNA from the two-hybrid yeast strain by a rapid isolation method such as the E.Z.N.A.TM Yeast Plasmid Mini Kit (Omega Bio-Tek, Inc.).
 - ii. Transform into *E. coli* RR1 and select for ampicillin-resistant colonies.
 - iii. Replica-plate colonies to M9 minimal plates lacking leucine (11,14). RR1 cells have a mutated *leuB* gene, which is a homolog of the yeast *LEU2* gene present on the pGAD plasmids and can be functionally replaced by *LEU2*.
 - iv. Retransform the isolated plasmids into the two-hybrid bait strain.
 - v. Repeat step 5 of 3.1.2 to confirm that the newly transformed plasmids continue to give a positive two-hybrid signal.
3. Once a prey clone has been verified to give a positive two-hybrid signal, the insert in the pGAD is sequenced. For genomic yeast DNA inserts, it is sufficient to sequence the ends of the insert because the entire genomic sequence of *S. cerevisiae* is available. The junction of the insert that is fused in-frame with the GAD domain in the pGAD plasmid can be sequenced with the primer 5'-TTCGATGATGAAGATACC-3' and

the distal junction of insert and vector can be sequenced with the primer 5'-TGAAGTGAAGTTGCGGGG-3' (11).

3.2. Modifications of the yeast two-hybrid method

Similar to ubiquitin, SUMO interacts covalently and/or noncovalently with other proteins. In covalent interactions, an isopeptide bond is formed between the glycine at the C-terminus of mature SUMO and a lysine side chain of the substrate protein; in noncovalent interactions, the most common interface involves residues in the β 2 strand of SUMO that bind to a stretch of aliphatic amino acids (usually followed by a cluster of acidic residues) known as a SUMO-interacting motif (SIM) on the interacting protein (10,15). Mutating SUMO residues involved in either type of interaction can help determine the nature of the interaction responsible for the two-hybrid signal. There are two desumoylating enzymes/SUMO proteases in yeast, Ulp1 and Ulp2, which are responsible for cleaving the isopeptide bond between SUMO and its substrates (16). Ulp1 also processes the SUMO precursor. Altering the expression level of either SUMO protease changes the profile of bulk SUMO-protein conjugates within the cell, which can be visualized by anti-SUMO immunoblotting (16) (*see* Figure 2). Moreover, altering SUMO protease expression levels can modulate the outcome of yeast two-hybrid interaction assays (9). A great advantage of the two-hybrid system compared to other methods of determining SUMO-interacting proteins is that by incorporating a few simple adaptations to the two-hybrid system, the type of interaction that is required between SUMO and the interacting protein can easily be determined.

Subheadings 3.2.1.–3.2.2 discuss various mutations incorporated in the pGBD-SUMO to determine if the SIM-binding domain or the conjugation site (or both) of the SUMO moiety is essential for a particular two-hybrid interaction. Refer to Figure 3 for a diagram of these potential interactions. In **Subheading 3.2.3**, the use of high-copy *ULP1*, which reduces the levels of most SUMO conjugates, is described as an additional way to test possible covalent interactions in the two-hybrid assay system.

3.2.1. Modifications to pGBD-SUMO that abolish its ability to conjugate to substrates—Once a protein is determined to interact with SUMO, the following modifications can be incorporated into the two-hybrid system. First, instead of using the standard pGBD-SUMO plasmid, a sequence encoding a SUMO mutant that lacks the last two glycine residues, is cloned into the pGBD plasmid, creating pGBD-SUMO Δ GG (9). SUMO Δ GG is not capable of covalently modifying a protein (17). Therefore, if a two-hybrid signal is still present with the expression of pGBD-SUMO Δ GG, SUMO must interact noncovalently with the protein (9) (*see* Note 3). Figure 4 compares two yeast SUMO-interacting proteins, one that interacts noncovalently (Ris1) and the other that is likely to depend on a covalent SUMO-protein interaction for strong interaction (Yen1).

3.2.2. Modifications to pGBD-SUMO that abolish noncovalent interactions—Structural and mutagenesis studies demonstrated that the β 2 strand of SUMO and part of the α helix are the chief sites of noncovalent binding to a substrate SIM (18,19,20). When mutations in these two regions were incorporated into a SUMO derivative expressed in the yeast two-hybrid system, interaction with a SIM-containing prey protein was greatly impaired; mutations with strong effects included the following: I34E, V38K, K39A, and K45/46A (20).

³In a complementary approach, it is possible to determine the site of covalent sumoylation by mutating lysine residues of the substrate in the context of the GAD-protein fusion. Often, sumoylation sites in proteins fit the consensus sequence hKxD/E (where h is a hydrophobic residue and x is any residue), providing a rapid means to identify potential SUMO attachment sites. When a lysine residue necessary for sumoylation of the substrate is mutated (usually to an arginine), the two-hybrid signal should be abolished. A limitation here is that a protein may be sumoylated on a number of different lysine residues, so identifying the relevant lysines will require making multiply mutant alleles.

Quikchange (Stratagene) site-directed mutagenesis can be used to mutate the pGBD-SUMO plasmid with any of the aforementioned mutations. The SIM-binding mutants are then used with the identified GAD-prey fusions to determine if SUMO-binding is inhibited. GAD-fusion peptides that produce a two-hybrid signal with the mutant GBD-SUMO probably do not need a SIM domain to interact with SUMO. It is noteworthy that noncovalent SIM binding is sometimes a prerequisite for covalent SUMO modification of the same protein (21). The core consensus SIM has been determined as I/V-X-I/V-I/V or the inverted motif and is usually flanked on one side by a cluster of acidic residues; however, the full range of functional sequence variations of SIMs has not been determined (15,22). As a complementary approach, if a putative SIM is identified in a prey protein, it can be mutated in the context of the pGAD plasmid to determine if it abolishes the two-hybrid signal.

3.2.3. Over-expression of ULP1—When the highly active and broad-specificity desumoylating enzyme Ulp1 is expressed from a high-copy plasmid, the level of SUMO conjugates is greatly reduced, as determined by Western blot analysis (*see* Figure 2) (12) (*see* Note 4 & 5). When *ULP1* is over-expressed in the yeast two-hybrid system, this decrease in bulk SUMO conjugates will often be paralleled by a loss of two-hybrid signal if the prey protein interaction with SUMO requires covalent SUMO modification (I, II in Figure 3). A previous two-hybrid screen showed a close correspondence between interactions that were sensitive to over-expressed *ULP1* and those that were sensitive to deletion of the terminal diglycine motif in SUMO (GBD-SUMOΔGG) (9). However, the concordance was not 100%, presumably because Ulp1 may not be active against some sumoylated proteins or the diglycine motif of SUMO may be necessary for certain noncovalent SUMO-interactions.

3.3. Verifying the sumoylation of a protein by a SUMO mobility shift assay

After a potential substrate has been identified by yeast two-hybrid analysis, the sumoylation of the substrate *in vivo* should be confirmed by an independent method. One relatively simple method is to look for forms of the substrate that migrate more slowly on an SDS gel than the unmodified protein and determine whether migration of these species is retarded further if cells express an epitope-tagged (larger) SUMO protein. Additions of peptide extensions to the N-terminus of SUMO are tolerated, although some are conjugated less efficiently than untagged SUMO. If antibodies to the target protein are unavailable, epitope-tagged derivatives can be generated. For yeast proteins, a library of genomically tagged ORFs is commercially available (Open Biosystems) in which individual yeast proteins are fused to a tandem-affinity purification (TAP) tag composed of a protein A segment and a calmodulin-binding peptide (23). If the protein is sumoylated to a significant level *in vivo*, Western blot analysis of whole cell lysates of logarithmically growing yeast should detect a slower migrating band(s), often present at a small fraction of the level of the unmodified protein (*see* Note 6).

In order to determine if the slower migrating species results from sumoylation of the target protein, the strain is transformed with either a plasmid that expresses high levels of untagged SUMO or a peptide-tagged SUMO derivative such as Flag-TEV-SUMO (FT-SUMO) (9). The Flag-TEV tag is large enough to cause a detectable supershift of the sumoylated species by Western blotting analysis for most proteins when compared to cells expressing untagged SUMO. Figure 5 provides an example. Note that endogenous SUMO is still present in these

⁴Very high levels of Ulp1, such as those generated by expression of *ULP1* from a galactose-inducible promoter, are toxic to the cell (12).

⁵Deleting *ULP2* or mutating *ULP1* (which is essential for viability) greatly increases the levels of specific SUMO conjugates and consequently might facilitate identification of certain two-hybrid interactors when such alleles are introduced into the two-hybrid yeast tester strain.

⁶Usually only a very small percentage of a substrate protein, often less than 1%, is sumoylated. This makes detection of its sumoylation difficult. Mutating the *ULP* genes or over-expressing SUMO can enhance detection sensitivity.

cells and is often conjugated more efficiently than the tagged SUMO, so both tagged and untagged SUMO derivatives are usually seen conjugated to the substrate (lane 3 in Figure 5).

3.4. Comparison of mass spectrometry and yeast two-hybrid methods for identifying SUMO binding proteins

Tandem mass spectrometry (MS/MS) is among the most widely used techniques employed to identify sumoylated substrates (24). Purification of epitope-tagged SUMO and its associating proteins followed by mass spectrometric analysis has yielded a far greater number of potentially sumoylated proteins compared to any other method, including the two-hybrid approach. One difficulty with the MS/MS approach, however, is that purified SUMO conjugates will be greatly enriched for substrates that are highly abundant even if they are only sumoylated to a very minor extent. This increases the probability of identifying proteins for which sumoylation occurs at very low frequency but is of little or no physiological consequence. Because expression of all two-hybrid clones in a given prey library is driven by the same regulatory sequences, biases in protein identification due to large differences in protein levels are substantially reduced. It is also generally necessary to purify SUMO-protein conjugates under stringent denaturing conditions for identification by MS/MS because such conditions inactivate SUMO proteases and because the level of copurifying contaminants would otherwise be unacceptably high. The drawback is that noncovalent SUMO-protein interactions are also largely eliminated.

Nevertheless, MS/MS and two-hybrid approaches should be regarded as complementary approaches, and substrates identified by either approach can be verified or further studied by the alternative method. For example, sumoylated proteins identified by MS/MS could be verified by the two-hybrid method and further analyzed by the modifications to the two-hybrid technique described above. Acute environmental changes and their effects on SUMO-conjugate profiles are more easily studied by MS/MS, but chronic stimuli or stresses, such as growth on different carbon sources, can be profitably studied by either method.

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Figure 1. Diagram of yeast two-hybrid plasmids

(A) Structure of the pGBD-SUMO and pGAD-C(x) plasmids. Stippled regions indicate the constitutive *ADHI* promoter (P) and transcription termination (T) elements. The sequence encoding mature yeast SUMO or its variants, indicated by the black segment, was cloned downstream of and in-frame with the sequence encoding the Gal4 BD (DNA-binding domain). In the pGAD plasmids, the multiple cloning site follows directly after the *GAL4* AD (activation domain) sequence. (B) Sequences of the multiple cloning region for the pGAD-C(x) and pGBDU-C(x) plasmids (11). Restriction sites are underlined; stop codons are boxed. The complete sequences of the pGAD plasmids can be found in Genbank under the accession numbers U70024 (pGAD-C1), U70025 (pGAD-C2), and U70026 (pGAD-C3). pGBD-SUMO is a derivative of pGBDU-C1, accession number U70021.

Figure 2. Comparison of bulk SUMO conjugates in different yeast strains

Anti-SUMO immunoblot of whole cell lysates of the following strains: (lane 1) wild type strain (WT); (lane 2) WT strain harboring a high-copy (HC) *ULP1* plasmid; (lane 3) *ulp1ts* mutant strain; and (lane 4) *ulp2Δ* strain. Strains were harvested during logarithmic growth at 30°C. For the lower panel, the membrane was reprobed with anti-Pgk1 to compare protein loading. * indicates high molecular weight polySUMO conjugates within the stacking gel.

A **B**

Variation of the yeast-two hybrid	Interactions identified
Standard	I, II, III & IV
GBD-SUMO Δ GG	III & IV
GBD-SUMO SIM binding mutant	I & II
ULP1 over-expression	Decrease in I & II
Compromised ULP	Increase in I & II

Figure 3. Diagram of potential SUMO-protein interactions identified from various permutations of the yeast two-hybrid assay

(A) Four ways by which pGBD-SUMO can interact with a GAD-prey fusion to generate a two-hybrid signal. (I) SUMO is covalently conjugated to the GAD-prey fusion. (II) SUMO is conjugated to a protein that associates noncovalently with the prey. (III) Direct noncovalent SUMO interaction with the prey. (IV) Noncovalent SUMO interaction with a protein that associates noncovalently with the prey. (B) Table listing the type of SUMO-protein interactions that can be identified by variations of the yeast two-hybrid system. See main text for details.

Figure 4. Conjugationally competent SUMO is necessary for some SUMO-protein interactions
 Two-hybrid fusion protein interactions activate the *GAL1-HIS3* reporter gene, allowing for growth on media lacking histidine. Either pGBD-SUMO or pGBD-SUMO Δ GG is expressed in the two-hybrid strain with one of the indicated pGAD-yeast gene fusion constructs. GAD-Yen1 can only interact with the conjugationally competent GBD-SUMO, whereas GAD-Ris1 interacts with both GBD-SUMO and GBD-SUMO Δ GG as determined by growth on medium lacking histidine.

Figure 5. SUMO mobility shift analysis of TAP-tagged Rap1

Immunoblotting of whole lysates from *RAP1-TAP* strains harboring (lane 1) the pRS426 empty vector; (lane 2) pRS426-SUMO; or (lane 3) pRS426-Flag-TEV-SUMO (FT-SUMO). Anti-PAP antibody, which recognizes the protein A segment within the TAP tag, was used.