transformant was applied to immunoprecipitation and Western blot analysis. The anti-Gts1p antibody against both immunoprecipitates recognized the Lsr1p-type only (Figure 2A). Amino acid (aa) composition analysis, on the purified Gts1p expressed in E. coli, also indicated that the Lsr1p-type composition is more likely than that of the Gts1p-type (8). Thus, we concluded that the Lsr1p-type composition of Gts1p (i.e., Lsr1p) has shown that the C-terminal half plays an essential role for flocculation (data not shown). Though the Myc-tagged Lsr1p showed fully functional flocculation property compared to that of intact Lsr1p, and the Myc-tagged Sfl1p showed the same results (data not shown). In this case, these two proteins were functional whether or not the Myc-tag is connected in their C terminus.

In this report, we describe the construction of a novel small epitope (Myc)-tagging E. coli-S. cerevisiae shuttle-vector system that is useful in the following ways: (i) relatively small-sized (4.3–6.2 kb) as a shuttle vector for yeast; (ii) five to six unique restriction enzyme sites in its MCS; (iii) four different selective markers (TRP1, HIS3, LEU2, and URA3); and (iv) two types of promoter (ADH1 and GAL1–10). It is hoped that this system will be a convenient tool for yeast researchers by eliminating the time-consuming antiserum generation.

Figure 2. Re-evaluation of the primary structure of Gts1p and detection of Myc-tagged Sfl1p. (A) Western-blot analysis with polyclonal anti-Gts1p antibody. Arrowhead indicates polypeptides recognized with anti-Gts1p antibody (4). (B) Western-blot analysis with 9E10 MAb antibody (MBL, Tokyo, Japan) (see Table 2).

REFERENCES


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Characterization of Tubulin Isotype-Specific Antibodies by Electrophoretic Mobility Shift Assay


Here, we present a novel biochemical application based on electrophoretic mobility shift assays for isotype-specific antibody evaluation. Native gel electrophoresis, which has been widely used to study protein-protein interactions (6) and the folding of newly synthesized proteins (9), allows the preservation of antibody-protein complexes formed during the incubation reaction. The resulting immuno-complexes will behave differently on electrophoresis than the actual protein, thereby resulting in a shift of the protein band along the gel run that serves to determine a positive antibody-binding reaction.

The existence of gene families is a feature of evolution. Gene families also result in families of proteins differing by only a few amino acid residues. The α and β-tubulin represent some of the best examples of gene/protein families. There are at least six α- and six β-tubulin genes, apart from several nonfunctional pseudogenes, expressed in vertebrates. Within either the α- or the β-tubulin families, each polypeptide differs from the others in less than 20% of its amino acid residues. This results in at least six different protein isotypes for each family, differing mainly in small amino acid sequences at their C terminus. This is an evolutionarily conserved gene family are often very difficult to characterize in terms of isoforms (6). Monoclonal or polyclonal antibodies against the type specificity. This is the case of iso-kinase site available (3,7). Moreover, small putative antibody-specific reactions were risen against proteins that belong to a family. Monoclonal or polyclonal antibodies are produced against proteins that belong to a conserved gene family are often very difficult to characterize in terms of isoform specificity. This is the case of isotype-specific antibodies against the tubulin protein family, where only a very small putative antibody-specific recognition site is available (3,7). Moreover, α- and β-tubulin polypeptides are subjected to many different posttranslational modifications, including acetylation, tyrosination, polyclaylation, polyglutamylation and phosphorylation (for a review, see Reference 5), which result in a high degree of heterogeneity (at least 21 charge variants) as observed by isoelectric focusing (IEF) (1,2). IEF is currently

### Table 1. Murine β-Tubulin Isotypes

<table>
<thead>
<tr>
<th>Class</th>
<th>Isotypes</th>
<th>C Terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>Mβ1</td>
<td>AGLEDSEDEAEEAEEVEAEDKDH</td>
</tr>
<tr>
<td>II</td>
<td>Mβ2</td>
<td>ADEQGFEEEEGEDEA</td>
</tr>
<tr>
<td>IVB</td>
<td>Mβ3</td>
<td>AEEGFEDEEAEEVA</td>
</tr>
<tr>
<td>IVA</td>
<td>Mβ4</td>
<td>AEEGFEDEEAEEVA</td>
</tr>
<tr>
<td>I</td>
<td>Mβ5</td>
<td>AEEEDFGEEAEEEA</td>
</tr>
<tr>
<td>II</td>
<td>Mβ6</td>
<td>AEEEGMYEDDEDESERQGPK</td>
</tr>
</tbody>
</table>

The existence of gene families is a feature of evolution. Gene families also result in families of proteins differing by only a few amino acid residues. The α- and β-tubulin represent some of the best examples of gene/protein families. There are at least six α- and six β-tubulin genes, apart from several non-functional pseudogenes, expressed in vertebrates. Within either the α- or the β-tubulin families, each polypeptide differs from the others in less than 20% of its amino acid residues. This results in at least six different protein isotypes for each family, differing mainly in small amino acid sequences at their C terminus. This is an evolutionarily conserved gene family are often very difficult to characterize in terms of isoform specificity. This is the case of isotype-specific antibodies against the tubulin protein family, where only a very small putative antibody-specific recognition site is available (3,7). Moreover, α- and β-tubulin polypeptides are subjected to many different posttranslational modifications, including acetylation, tyrosination, polyclaylation, polyglutamylation and phosphorylation (for a review, see Reference 5), which result in a high degree of heterogeneity (at least 21 charge variants) as observed by isoelectric focusing (IEF) (1,2). IEF is currently

the most common technique for the identification of the different charge variants, although it is difficult to correlate different IEF bands with the corresponding isotype. A more labor-intensive alternative to this method would be to perform Western blots of single isotypes expressed in bacteria. However, this procedure would only serve for the study of denatured expression products that in addition might present a proteolyzed C terminus due to unspecific bacterial proteases.

Two polyclonal antibodies produced in our laboratories against the C-terminal peptides EGEDEA and EMYEDDEEESQGPK of the β2- and β6-tubulin isotypes were used during the study. The isotype specificity of these antibodies was tested against the six different β-tubulin isotypes (Table 1). These had been individually translated and labeled in vitro using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) in the presence of 35S-methionine (>1000 Ci/mmol; Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting reactions, containing radiolabeled newly synthesized β-tubulin polypeptides, were incubated with the two antibodies in separate experiments. Incubation reactions were then electrophoresed under non-denaturing conditions. This allows the identification and separation of the different molecular forms, which are generated at the progressive stages of the tubulin folding pathway as previously described (9). These are monomers (Figure 1), αβ-heterodimers and other tubulin multimolecular complexes that also enter the gel. Antibody binding to

![Figure 1. Tubulin isotype-specific antibodies.](image)

Anti-β2 and anti-β6 affinity for the six different murine tubulin isotypes studied on in vitro translation products of the respective tubulin genes. Reaction products run under non-denaturing conditions resulted in several major bands, monomers (M) of the proteins bound to the p14 chaperone (4), αβ heterodimers (D) and higher molecular weight complexes (not indicated) (9). Antibody binding to in vitro-translated proteins formed larger complexes that could be recognized by a shift or disappearance of the protein band in the gel. Translation reactions of different tubulin isotypes were incubated with (+) or without (-) anti-β2-tubulin antibody (upper panel) or the anti-β6-tubulin antibody, U-β6 (lower panel). A clear retardation that was observed only when β2-tubulin products were incubated with anti-β2-tubulin antibody, demonstrated the isotype specificity of this polyclonal antibody. The monomer disappearance observed when β6- and β1-tubulin products were incubated with U-β6 with respect to other isotypes, demonstrated that U-β6 recognized mainly β6-tubulin monomers and also partially cross-reacted with the platelet-specific isotype β1.
any of these proteins or their complexes during the posttranslation incubation period would produce larger molecular immuno-complexes than the actual forms, and thus would result in a delay of the progression of these proteins through the gel, identifiable by a shift or disappearance of the protein band.

A total of 0.4 µL of the antiserum or control pre-immune rabbit serum was incubated with 3 µL of the above in vitro reaction products, plus 16.6 µL of Buffer A (100 mM MES, pH 6.6, 1 mM MgCl₂; Sigma Chemical, Madrid, Spain) containing 1 mM GTP (Boehringer Mannheim GmbH, Mannheim, Germany). Inclusion of GTP in the reaction buffer was required for the stabilization of the monomeric and dimeric forms of tubulin (9). Incubation was performed at 4°C for 90 min with shaking every 15 min. Immediately after antibody incubations, 4 µL of loading buffer (25% sucrose, 50 mM MES, pH 6.6) were added to the samples. These products were then loaded onto 4.5% polyacrylamide native gels prepared and run as previously described (9). Following electrophoresis, gels were fluorographed, dried and exposed overnight to film (Hyperfilm™ MP; Amersham Pharmacia Biotech) at -70°C. Quantification was carried out using a Model GS-363 Molecular Imager System (Bio-Rad, Hercules, CA, USA).

Resulting fluorographs revealed patent differences at the lanes in which antibodies had been incubated with the β-tubulin isotypes they were raised against (Figure 1). However, the anti-β2-tubulin antibody appeared more specific (Figure 1, upper panel) than U-β6, which also recognized the β1-tubulin isotype (Figure 1, lower panel). The radioactivity at the monomer bands obtained for all isotypes were quantified. Obtained data confirmed that the anti-β2 antibody incubated with the β2-tubulin resulted in more than 95% disappearance of monomers. The reduction observed for the β1, β3, β4, β5 and β6 monomer bands incubated in parallel, with the same antibody, was less than 5%. U-β6 was highly specific against the β6 isotype, though this antiserum also presented some affinity for the platelet-specific, β-tubulin isotype β1 (8), resulting in a 35% radioactivity reduction at the β1-tubulin monomer band. Although β6 is apparently more similar to β2 than β1, this last isotype shares with β6 a common element at their C terminus (an acidic residue followed by a basic residue) that might result in antibody cross-reaction.

In conclusion, this isotype antibody-typing method allows not just the qualitative, but also the quantitative analysis of antibody-isotype specificity, combining a powerful technique such as non-denaturing electrophoresis with the capability to quantify in vitro-labeled products, by using molecular imaging systems.

REFERENCES


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