Binding Specificity of Protein Phosphatase 2A Core Enzyme for Regulatory B Subunits and T Antigens

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The core enzyme of protein phosphatase 2A is composed of a regulatory subunit A and a catalytic subunit C. It is controlled by three types of regulatory B subunits (B, B′, and B″) and by tumor (T) antigens, which are unrelated by sequence but bind to overlapping regions on the A subunit. To find out whether the different B subunits and T antigens bind to identical or distinct amino acids of the A subunit, mutants were generated and their abilities to bind B subunits and T antigens were tested. We found that some amino acids are involved in the binding of all types of B subunits, whereas others are specifically involved in the binding of one or two types of B subunits. T-antigen-binding specificity does not correlate with that of a particular type of B subunit.

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Protein phosphatase 2A (PP2A) is an abundant enzyme constituting approximately 0.3% of the total protein in mammalian cells (17). It exists in cells in two forms—the core enzyme, composed of a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit, and the holoenzyme, consisting of core enzyme to which one of several B subunits is bound—at approximately equal concentrations (8). The A and C subunits both occur as two isoforms (α and β), whereas the B subunits fall into three families, designated B, B′ (also called B56), and B″, that are unrelated by protein sequence (13). The B family has three members, Bα, Bβ, and Bγ, each with a molecular mass of around 55 kDa (5, 10, 14, 23). The B′ family consists of numerous, recently identified isoforms and splice variants whose molecular masses range from 54 to approximately 70 kDa (3, 11, 12, 18, 19). The B″ family has two members, which have molecular masses of 72 and 130 kDa and are splice variants of the same gene (7). B subunits are the key regulators of PP2A. They determine not only the activity and substrate specificity but also the intracellular localization of PP2A (11, 19). In addition to the three classes of B subunits, a fourth class of proteins that associate with the PP2A core enzyme are the regulatory subunits B, suggesting that there was no specificity in binding of all other B subunits and of T antigens. (ii) B subunits and T antigens recognize distinct amino acids. This model predicts that one could generate mutants of the A subunit that are unable to bind certain B subunits or T antigens while still binding others. Our previous data supported the first model, since deletion of any one of repeats 1 to 10 or substitution of intrarepeat loop 4, 5, or 6 abolished binding of all types of B subunits, suggesting that there was no specificity in binding of individual B subunits. On the other hand, the first model seemed unlikely because the same binding site would have to have evolved in four unrelated protein families.

To resolve this question, we generated smaller mutations by substituting for only 1 to 4 amino acids in intrarepeat loops 3 (amino acids 100 to 105), 4 (amino acids 139 to 144), 5 (amino acids 177 to 182), and 7 (amino acids 255 to 260). Some...
mutations outside the loop regions were also generated. The expectation was that smaller mutations might have a better chance of revealing differences in binding specificity between different B subunits and T antigens. Site-directed mutagenesis was performed with the Gene Editor system from Promega. The plasmid used for mutagenesis, pcDNA3-AEE, is a eukaryotic expression vector encoding the wild-type A subunit tagged at the C terminus with the EE tag (EEEEYMPME) (9). The tag was introduced into the A subunit within Bluescript, p161 (21), by insertion of a fragment generated by PCR and encoding a BglII site, the EE tag, and a HindIII site. AEE was then moved from Bluescript to pcDNA3 as an EcoRI-XhoI fragment. To assay whether A subunit mutants bind B subunits, both were synthesized separately in vitro and labeled with \(^{35}S\)methionine by using Promega’s TNT T7 Quick coupled transcription/translation system, as previously described (8). Five microliters of each reaction mixture was combined and incubated for 4 h at 30°C to allow complex formation between the labeled A subunit mutant, endogenous C subunit (unlabeled), and labeled B subunit, as previously described (15, 16). Anti-EE monoclonal antibodies were used to immunoprecipitate the tagged A subunit mutants. The precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify coprecipitated B subunits. Since in
vitro-synthesized middle T does not form a complex with core enzyme (1), we used a previously described assay to determine whether A subunit mutants bind T antigens (16). The mutants were synthesized in vitro as described above. SV40 small t was added as a bacterially expressed purified protein (20), polyomavirus small t was added as a baculovirus-expressed protein in Sf9 cell lysate, and middle T was added as an overexpressed protein in 293 cell lysate. Hamster antitumor ascitic fluid was used to precipitate polyomavirus small t and middle T (16). The mixtures were incubated at 4°C for 16 h. The immunoprecipitates were washed with buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100) and analyzed by SDS-PAGE. Coprecipitated A subunit mutants were visualized by autoradiography. Radioactive bands were quantitated with a Molecular Dynamics PhosphorImager and ImageQuant software.

The results demonstrate that some mutations affect binding of all B subunits, whereas others affect binding of specific B subunits (Fig. 2 and 3). The mutants fall into four categories. (i) \( B^+ B^- B^* \). An example is mutant EE100RR, which bound none of the B subunits.

(ii) \( B^+ B^- B^* \). An example is mutant DW139RR, which showed almost no binding of \( B^o \) and \( B^*- \) whereas binding of \( B^* \) was reduced only by one-third.

(iii) \( B^+ B^- B^* \). An example is DWF139HAA, which bound \( B^o \) as well as or better than the wild type but did not bind \( B^- \) or \( B^* \). Note the dramatic difference in specificities between W140A (\( B^+ B^- B^* \)) and DWF139HAA (\( B^+ B^- B^* \)), indicating that W140 is essential for \( B^- \) and \( B^* \) binding and F141 is required for \( B^* \) binding. D139 had little if any effect, since the binding patterns of DW139RR and W140A are very similar. It is intriguing that mutation of W140 destroyed binding of \( B^o \) and that the additional mutation of F141 restored binding of \( B^* \).

(iv) \( B^+ B^- B^* \). An example is DTP177AAA, which showed a 25-fold reduction in binding of \( B^- \) whereas binding of \( B^o \) was normal and that of \( B^* \) was reduced approximately 50%. This mutant is most specific because its deficiency is limited mainly to one type of B subunit (\( B^- \)). In view of the fact that the binding regions for all B subunits extend over 10 repeats, it is remarkable that a point mutation such as W257A in repeat 7 completely destroyed the binding capacity for all B subunits (but not for T antigens).

With regard to T antigens, our data demonstrate that, with the exception of EE100RR, mutations that reduced binding of one T antigen also reduced binding of the others. Polymavirus middle T was more affected than the small t antigens. This is consistent with our previous findings (15, 16). In general, the amino acids involved in binding T antigens were also located in intrarepeat loops. However, one mutant, L166E, located outside of intrarepeat loop 5, showed reduced binding of T antigens but had no effect on B subunit binding. We previously identified a region (intronrepeat loop 4/5, amino acids 158 to 165) very close to L166 that is also involved in binding polyomavirus middle T and small t only (15) (Fig. 1). Our results reveal no apparent correlation between the binding of T antigens and the binding of a particular B subunit.

The reason why EE100RR binds SV40 small t but not polyomavirus small t and middle T is probably related to the fact that the binding region for SV40 small t involves repeats 3 through 6 and that for polyomavirus T antigens involves repeats 2 through 8. Probably, the binding region for SV40 small t starts downstream of E100.

We conclude that the binding sites on the A subunit for the different types of B subunits are composed of both distinct and common amino acids. Our results also indicate that all mutations that affect B subunit binding are located in intrarepeat loops, since no mutation outside of these regions had a significant effect on binding. This finding further supports the role of intrarepeat loops in binding, as suggested by our model of the A subunit (Fig. 1) (15, 16). Mutants of the A subunit defective in the binding of specific B subunits are potential tools for studying the role of PP2A in vivo. For example, substitution of the wild-type A subunit by gene replacement with a mutant that is defective in B’ but normal in B and B’ binding may shed light on the function(s) of B’ subunits. Furthermore, the mutant L166E, which is partially defective in binding middle T but not normal in B subunit binding, could be used to study the role of the middle T-PP2A interaction in tumor formation. Whereas the binding of PP2A to middle T is a prerequisite for transformation of cultured fibroblasts (1, 4), its importance for tumor formation has not yet been demonstrated. We plan to generate mice in which the wild-type A subunit is replaced by the mutant L166E. If the binding of middle T to PP2A is important, these mice should be immune to polyomavirus- or middle T-induced tumors.

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REFERENCES
