

Protein-Protein Interactions Between the Multifunctional Protein CAD and Protein Phosphatase 1

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The supply of pyrimidine nucleotides in mammalian cells is regulated by a large multifunctional protein called CAD. The activity of the protein invariably increases in tumor cells to meet the increased demand for pyrimidines during proliferation. The CAD complex is activated by MAP kinase just prior to the S phase of the cell cycle, when DNA synthesis occurs. MAP kinase is an enzyme that phosphorylates proteins involved in cell growth and division. Growth signals initiate a signaling cascade that activates MAP kinase and leads to the phosphorylation of CAD. Upon completion of DNA synthesis, the cells emerge from S phase, CAD is dephosphorylated by a phosphatase called PP1 and a second kinase, protein kinase A, phosphorylates a different site on the complex leading to deactivation and a decrease in the flux through the *de novo* pyrimidine biosynthetic pathway. The timing of the up- and down-regulation of CAD must be very precise but the control mechanisms that regulate the process are not understood. Our hypothesis is that the timing is controlled by the formation of dynamic complexes of CAD with MAP kinase, PP1 and PKA.

My project focuses on the interactions between CAD and PP1. PP1 is known to bind to many different targeting proteins. The sequence recognized by PP1 at the binding site of all of the targeting proteins is highly conserved, Lysine (or arginine)-Valine-X-Phenylalanine where X is any amino acid. There is only one such sequence in the CAD complex and it is located immediately adjacent to the MAP kinase site, Thr456.

The working hypothesis is that phosphorylation of Thr456 blocks the binding of PP1 and thus prevents the premature dephosphorylation and down-regulation of the pathway. At the end of S phase, the phosphorylation of CAD by PKA alters the conformation of the protein allowing dephosphorylation of Thr456 by PP1. My project has two goals. The first goal is to determine whether or not we have correctly identified the site on CAD that binds PP1. The second goal is to determine whether the binding of PP1 depends on whether or not Thr456 is phosphorylated. The rationale for this project is that it would provide a significant insight into the timing mechanism and perhaps suggest chemotherapeutic interventions.

Several different methods were employed to address the goals of the project. Immunoprecipitation was used to confirm that a complex forms between CAD and PP1. In this method, one of the proteins, e.g. CAD are reacted with specific antibodies and then incubated with beads that have a high affinity for immunoglobulins. The protein bound to the bead is then reacted with a second antibody, in this case directed against PP1 to see if it is also present in the complex bound to the beads. To determine the location of the PP1 binding site on CAD, I synthesized a peptide in collaboration with Dr. Debbie Heyl-Clegg, which corresponds to the naturally occurring sequence in CAD.

444 Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Ile-**Thr**-Pro-His-Tyr-Val 460
putative PP1 binding MAP kinase site

The peptide was used in competition experiments to determine whether it binds to PP1. There is a PP1 inhibitor in the cell called inhibitor 2 that binds to the same consensus sequence. Inhibition of PP1 was measured by assaying the hydrolysis of p-nitrophenyl phosphate, which produces a colored product that can be measured spectrophotometrically. If the peptide binds to PP1, it should block the inhibition by inhibitor 2. Finally, to determine whether the phosphorylation state of Thr456 influences the interaction with CAD, I synthesized two additional peptides. In one, Thr456 was replaced with Ala, which cannot be phosphorylated, while in the second peptide, Thr456 was replaced with aspartate. The aspartate carries a negative charge and mimics the effect of constitutive phosphorylation.

The *outcome* of the experiments described for *goal 1*, was that the synthetic peptide prevented the binding of inhibitor-2 to PP1. This result provides good evidence that we have located the PP1 binding site on CAD, an interpretation that was confirmed by the *outcome of goal 2*. The Thr456Ala mutant, like the wild type peptide, binds to PP1, but the Thr456Asp mutant does not. The results confirm our expectations and support one aspect of our working hypothesis; there is a PP1 binding site on CAD near the MAP kinase phosphorylation site. Whether or not PP1 actually binds to this site depends on whether the complex has been phosphorylated by MAP kinase. An unanticipated result is that when PP1 binds to CAD, the phosphatase activity increases more than 2-fold. While we do not as yet understand the significance of this finding, it may prevent premature phosphorylation of the PKA site.

This project paves the way for *future studies* of these regulatory mechanisms. The next step is to investigate the interactions of PP1 with wild type CAD and the Thr456 mutants by co-immunoprecipitation experiments. We already have constructs that express CAD and the mutant proteins and anticipate that PP1 would bind to the wild type and Thr456Ala mutant but not the Thr456Asp mutant. We have preliminary evidence that PKA also binds to CAD. It will be interesting to determine whether PKA binding alters the interactions of CAD with PP1 and the phosphorylation of Thr456. We also plan to introduce the wild type and mutant peptides into permeabilized mammalian cells to determine by immunofluorescence microscopy, whether CAD-PP1 interactions occur in the cell.

The *unique contributions* of this project are that although the up- and down-regulation of the pathway has been well-established, there was no information about the mechanism that controls the process. This represents a significant gap in our understanding of pyrimidine regulation and is a bottleneck in the design of drugs that could interrupt the process in rapidly proliferating cells. This project, which demonstrates that CAD forms a regulated complex with PP1, represents the first step in deciphering these mechanisms.