PROTEOMIC INVESTIGATION OF THE MECHANISMS CONTROLLING THE CYCLIN D-DEPENDENT KINASE

Marco Crescenzi
Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy

This project has been carried out accordingly to the original proposal and it has yielded significant scientific results with great therapeutic potential.

Previous work from the PI's group has shown that the cyclin D-dependent kinase activity plays a critical role in the regulation of the postmitotic state of Terminally Differentiated (TD) cells. The first aim of the project was to unravel the molecular mechanisms that repress such kinase activity in TD cells. The use of complementary biochemistry and mass spectrometry techniques has allowed us to answer this question satisfactorily.

Skeletal muscle cells (myotubes) have been infected with recombinant adenoviruses expressing cyclin D1 and cdk4, the main kinase partner of D-type cyclins in the biological systems studied. cdk4-containing complexes have been immunoprecipitated and resolved by SDS-PAGE. The proteins precipitated along with cdk4 have been identified by combining mass fingerprint on a MALDI-TOF instrument and tandem mass spectrometry on a ESI-ion trap mass spectrometer. The cyclin-dependent kinase inhibitor p21 was among the proteins identified.

On the basis of these experiments, p21 expression has been suppressed by RNA interference (RNAi). Surprisingly, the sole ablation of p21 induced mitotic reactivation in 65% of the myotubes. When, along with p21, another member of the same family, p27, was also suppressed, more than 80% of the myotubes were reactivated. To understand the mechanisms through wich suppression of cdk inhibitors causes cell cycle reactivation, new immunoprecipitation-mass spectrometry experiments were carried out. Such experiments further dissected cdk4-containing complexes, showing that they contain mainly cyclin D3 and p21. When cyclin D3 was suppressed or a dominant negative mutant of cdk4 was expressed in p21-interfered myotubes, cell cycle reentry was abolished, showing that such reentry critically depends on cyclin D3/cdk4 complexes. An extensive biochemical characterization of myotubes has produced results in perfect agreement with this conclusion.

We then asked if other non-proliferative states are also maintained by and depend on cell cycle inhibitors. To address this question, human primary fibroblasts were put into quiescence by serum starvation and contact inhibition, then subjected to RNAi for various cell cycle inhibitors, among which p21, p27, and p18. Similar to myotubes, the fibroblasts thus treated were readily brought back into the cell cycle. Finally, senescent human fibroblasts or human embryo kidney cells were efficiently reactivated (up to 80%) by interfering with either p21 or p16. This result is particularly meaningful since senescent cells, like TD ones, are traditionally regarded as irreversibly non-proliferating.

Altogether, the experiments described thus far can be viewed as an example of successful application of modern proteomics techniques to solve an important biological problem. The results obtained have gone beyond expectations, allowing us to conclude that, in contrast with common belief, physiological non-proliferative states (terminal differentiation. quiescence, and replicative senescence) must be actively maintained and are not the result of the mere absence of pro-mitotic mechanisms and regulations.

The ability to stimulate or promote proliferation in a variety of cell types by simply suppressing cell cycle inhibitors in a temporary and reversible fashion has the potential to allow

the cultivation of cells endowed with limited replicative potential, among which many adult human stem cells, and accelerate *in vivo* tissue repair. Experiments on these subjects are in progress and the preliminary results are very encouraging.

The results described so far have been published (Pajalunga *et al*., 2007a) and their implications have been extensively discussed (Pajalunga *et al.*, 2007b; Pajalunga *et al*., 2008).

In the course of these studies, we were also able to describe a novel, significant mechanism controlling the mitotic cycle. It can be briefly described as a positive feed-back between E2F-family transcription factors and cyclin E. We have shown that E2F overexpression, frequent in human tumors, increases the half-life and hence the steady-state levels of cyclin E, inducing cell cycle deregulation. These results have been published (Pajalunga *et al*., 2004).

This project has been carried out in a collaboration with Dr. Ettore Appella (Chief, Chemistry Section, Laboratory of Cell Biology, National Cancer Institute-NCI) and Dr. Donald Bottaro (Urologic Oncology Branch, Center for Cancer Research, NCI). In particular, Dr. Anna Maria Salzano, a mass spectrometrist belonging to the PI's group, has spent one month in Dr. Appella's laboratory to refine the mass spectrometry techniques employed in this project. Dr. Deborah Pajalunga, a biologist also belonging to Dr. Crescenzi's group, carried out the first part of the project in Dr. Bottaro's laboratory, where she refined chromatography methods and techniques to measure affinity constants. Altogether, a three-pole collaboration has yielded results with high scientific and translational value.

The highly significant results obtained so far demand that the current collaborations are continued. The many possibilities to further develop these results, even into therapeutic approaches, require that the research efforts are supported in the future as well as possible.

Publications of the project

Pajalunga D, Crescenzi M. Regulation of cyclin E protein levels through E2F-mediated inhibition of degradation. *Cell Cycle* 2004;3:1572-8.

Pajalunga D, Mazzola A, Franchitto A, Puggioni E, Crescenzi M. Molecular and Cellular Basis of Regeneration and Tissue Repair: The logic and regulation of cell cycle exit and reentry. *Cell Mol Life Sci* 2008;65:8-15.

Pajalunga D, Mazzola A, Salzano AM, Biferi MG, De Luca G, Crescenzi M. Critical requirement for cell cycle inhibitors in sustaining nonproliferative states. *J Cell Biol* 2007a;176:807-18.

Pajalunga D, Mazzola A, Puggioni E, Crescenzi M. Non-proliferation as an active state: conceptual and practical implications. *Cell Cycle* 2007b;6:1415-8.