

Novel Means of Identifying Specific Proteins of Multisubunit Complexes

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OVERVIEW

Purpose

> Identification of novel interacting proteins of multisubunit complexes in yeast.

Methods

DIGE

> Immunoprecipitations were performed with Cdh1 harboring a 3X FLAG epitope in yeast. A control sample from the untagged parent yeast strain is used for comparison for DIGE experiments. The control sample is labeled with one fluorescent dye (cy3), and the tagged pull-down sample is labeled with the second dye (cy5).

> Samples separated on single 2D gel and imaged for each specific dye. Images are then overlaid to view specific interacting proteins.

Differential Chemical Modification

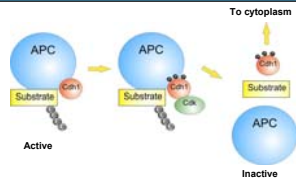
> Using differential modification, we use acetic anhydride to acetylate peptides from the typically digested control sample and a hexadeuterio derivative to modify digested target peptides. The two samples are combined, separated, and analyzed by LC/MS-MS.

Results

> Ten specific proteins to Cdh1-FLAG IP were detected initially in our DIGE experiment with four additional proteins detected compared to 1D gel separation.

> Differential chemical modification is effective in quantitating protein levels in blood plasma and can be applied to identify novel interacting proteins of APC^{Core}.

INTRODUCTION



The anaphase-promoting complex (APC) is an E3 ubiquitin ligase that regulates various cell cycle events. Cdh1 is a member of a family of proteins required for APC activity and believed to be involved in recruitment of specific substrates to APC^{Core}. The activity of APC^{Core} is regulated by cyclin-dependent kinase (CDK) phosphorylation of Cdh1 such that phosphorylated Cdh1 is unable to interact with APC, rendering it inactive^{1,2}. Cdh1 is not only tightly controlled by phosphorylation but also through interactions with other proteins. Although some of the proteins for regulation of APC and Cdh1 and targets for proteolysis are known, the list is not complete and a novel means of identifying these transiently interacting proteins is needed. Two powerful approaches using 2D differential gel electrophoresis (DIGE) and a differential chemical modification method in combination with mass spectrometry allow for the separation, detection, and identification of specific interacting proteins with the target of interest.

- References:**
1. Visintin, R., Prinz, S., and Amon, A. (1997) *Science* **278**: 460-3.
 2. Fang, C., Yu, H., and Kirschner, M.W. (1998) *Mol Cell* **2**: 163-71.
 3. Zachariae, W., Schwab, M., Nasmyth, K., Seufert, W. (1998) *Science* **282**: 1721-24.
 4. Jaspersen, S.L., Charles, J.F., and Morgan, D.O. (1999) *Curr Biol* **9**: 227-36.

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METHODS

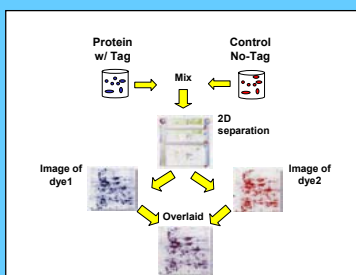


Figure 1 Strategy for DIGE

- Yeast Cdh1 protein was genomically tagged with three copies of the FLAG epitope.
- Cdh1-FLAG was immunopurified from whole cell extract using anti-FLAG antibody-coupled resin and eluted by competition with 3xFLAG peptide. Untagged isogenic parent strain used as control.
- A portion of the Control and tagged samples were separated by 1D gel electrophoresis and Coomassie stained for comparison with 2D DIGE.
- Cdh1-FLAG immunoprecipitation incubated with cy5 fluorescent dye and control with cy3 fluorescent dye.
- Samples combined and separated by single 2D gel.
- The gel is imaged at the specific wavelength for each dye and each image overlaid.
- In the pseudo-colored cy3 (magenta), cy5 (green) overlaid images, proteins present in both samples appear black and are non-specific, while green proteins are specific to the tagged pull-down sample.
- Excise proteins of interest, digest with trypsin and analyze by MALDI-TOF/TOF.

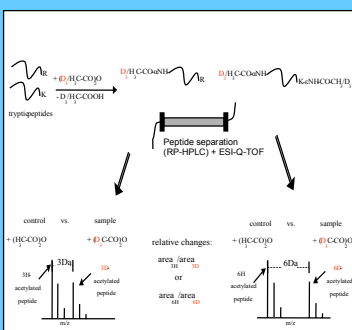


Figure 2 Strategy for Differential Chemical Modification

- Perform immunoprecipitation with selected samples.
- Digest control sample with trypsin and acetylate the peptides with acetic anhydride.
- Digest the epitope-tagged sample with trypsin and treat peptides with hexadeuterio derivative.
- Combine samples, separate by LC and analyze by electrospray quadrupole time-of-flight mass spectrometer.
- Peptides which are only present in the tagged sample will not have an acetylated counterpart from the control and can be considered specific to the tagged sample.

RESULTS

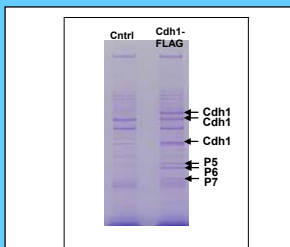


Figure 3 Identification of Novel Interacting Proteins From Cdh1 IP and 1D separation

• Using this separation technique, three novel proteins present in the Cdh1-FLAG lane and not in the control lane were identified.

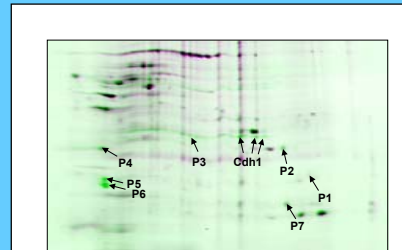


Figure 4 Separation and detection of proteins from Cdh1 IP by 2D DIGE

- Proteins specific to Cdh1-FLAG appear green while non-specific proteins present in both control and Cdh1-FLAG samples appear black.
- Using this technique, four additional proteins were able to be detected compared to conventional 1D separation and Coomassie staining.

RESULTS

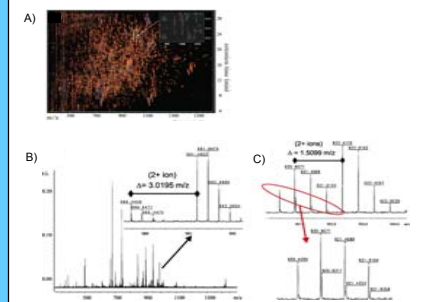


Figure 5 Relative quantification of protein expression level (human blood plasma) by LC-FTICR-MS after proteolysis and differential labeling using acetic anhydride vs. hexadeuterioacetic anhydride.

A) Heat-map of the LC-MS analysis with x-axis represents the m/z values of the ion signals observed in the MS and the y-axis the retention time of the LC-run. The intensity of the ion signals is color-coded. B) The inset depicts a magnified portion of the spectra showing doublets based on differential labeling. C) MS-spectra demonstrating the resolution acquired for accurate determination of doublets and the ion signal ratio which relates to the change in the protein expression.

CONCLUSIONS

- Use of the 2D-DIGE technique provides good separation and easy visualization of specific interacting proteins while enabling the user to run both the control and target samples on one gel. This eliminates the need for running two separate gels and the problem of reproducibility and aligning gels to view specific proteins. In addition, use of two different sensitive fluorescent dyes allows the detection of specific low-abundant proteins. Like all methods involving gel electrophoresis, DIGE is not useful for membrane proteins or those having extreme MW or pI. However, DIGE does allow enhanced separation and the detection of complex protein mixtures and isoforms while mass spectrometry provides the ability to unambiguously identify the proteins of interest.
- To complement DIGE analysis, differential chemical modification by acetylation provides a means for detecting specific proteins which are not suitable for separation by gel electrophoresis. The method requires little starting material, provides high sensitivity and dynamic range, and can be high throughput.
- While each method possesses certain advantages over the other, both provide novel means of detecting and separating specific proteins of multisubunit complexes.