6.1

The Study of Organelle Dynamics Using Stable Isotope Labeling

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Cells are organized spatially and functionally into sub-cellular compartments. Changes in sub-cellular localization are involved in regulation of interactions, stability and activity. Studying global changes in protein localization can provide useful insights into cellular functions.

We have previously developed LOPIT, a high-throughput technique for protein localization to subcellular organelles (1,2). Organelles are partially separated by density gradient centrifugation and fractions labeled with stable isotopic iTRAQ tags for quantitation. Proteins from the same organelle co-sediment and exhibit similar distributions in the density gradient. Sub-cellular localization can be assigned by comparing distributions of unknown proteins to those of known organelle markers. Using this approach we have been able to assign hundreds of proteins to different sub-cellular locations within Arabidopsis cultured cells.

We have now extended LOPIT to map protein localization within *Drosophila melanogaster* embryos and vertebrate cell lines. Data from these studies will be presented along with data which demonstrates that protein super-complexes such as the proteasome can also be mapped using this method.

We are currently using the LOPIT technique to dynamically map protein redistribution upon a given perturbation. The key to this process is to be able to control for technical variability within the LOPIT experimental schema, which otherwise may obscure genuine translocation of protein species between sub-cellular locations.

Methods to account for the technical variability associated with this approach will be introduced.

References

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6.2

Elucidating Interacting Regions within a Large Protein Complex through Chemical Crosslinking and Mass Spectrometry

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Recent developments in MS technologies have significantly reduced the amount of material required to fully analyze modified peptides, which has led to a resurgence of protein chemistry techniques, including chemical crosslinking. For proteins that are not readily amenable to X-ray diffraction analysis, such as large oligomeric complexes, the identification of crosslinked amino acids by MS provides an alternative approach for obtaining relatively high resolution structural information. Despite the resolving power of MS, however, considerable computational analysis of digests from crosslinked proteins is required to account for all the possible masses that can arise not just from intermolecular crosslinking, but also from intramolecular crosslinking, incomplete digestion, monoderivatization, and hydrolysis and other side reactions that can occur on the crosslinker itself. Consequently, assignment of masses from crosslinked digests is invariably the slowest step in this structural approach. To facilitate analysis and assignment, we have constructed a search engine that generates best matches for crosslinked peptides, based on the identity of the crosslinker and on theoretical peptide maps of the proteins in question as digested by a given protease. This engine was used to identify crosslinked residues among the various subunits of the large model oligomeric complex phosphorylase kinase (PhK).

PhK from skeletal muscle is a stable complex of sixteen subunits, $(\alpha\beta\gamma\delta)_4$, and a mass of 1.3 MDa. Its γ subunit is catalytic, while the remaining subunits are regulatory, giving rise to activation through such mechanisms as their phosphorylation or the binding of Ca^{2+} ions. In fact, 90% of PhK's mass is involved in its regulation. Thus, it has been of considerable interest to determine which regions of PhK's regulatory subunits interact with its catalytic γ subunit to control activity. The γ subunit contains 386 amino acids having a mass of 44.7 kDa and is composed of an N-terminal catalytic domain of ca. 286 residues and a basic C-terminal domain of ca. 100 residues that will be referred to as its regulatory domain, or γCRD. A variety of chemical crosslinkers, zero-length or short when possible, were screened to identify those that could form γ-δ, γ-β and γ-α dimeric conjugates when reacted with the hexadecameric $(\alpha\beta\gamma\delta)_4$ PhK complex. These dimers were subsequently fractionated and analyzed by MS, following tryptic digestion. A γ-δ dimer was formed by the zero-length crosslinker N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and was linked through an isoamide bond between K325 of the γCRD and D93 of the 16.7 kDa δ subunit, an intrinsic molecule of calmodulin. A γ-α dimer was selectively formed in large amounts by the very short crosslinker formaldehyde. Two-hybrid screening had initially indicated that the regions of interaction between these subunits occur somewhere within residues 343–386 of the γCRD and 1060–1237 of the 138.4 kDa α subunit, a region just C-terminal to a hyperphosphorylated sequence and that extends to the C-terminus of α. Our search engine identified peptides from digests of the γ-α complex that corroborate these regions of interaction; however, the data have yet to be fully analyzed to determine the residues crosslinked. A longer and more complex crosslinker, N-[γ-maleimidobutyryloxy]succinimide ester (GMBS), was required to form the γ-β conjugate. GMBS, which appeared to act as an affinity crosslinker, formed an unusual linkage between K303 of the γCRD and R18 of the 125.2 kDa β subunit. This residue near the N-terminus of β lies between its phosphorylatable seryl residues 11 and 26. Inasmuch as these residues are subject to intramolecular autophosphorylation by γ, the crosslinking of βR18 to γK303 also places the γCRD near the catalytic site of γ. The sum of our crosslinking results indicates that regulatory regions of the δ, α and β subunits of PhK directly interact with, or at the very least in the case of β lie near, the C-terminal regulatory domain of the catalytic γ subunit. The juxtaposition of these regions provides a physical rationale for the activation of PhK by Ca^{2+} (through the δ subunit) and phosphorylation (through the α and β subunits).

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