Lessons From Old and New Kinases

J.E. Van Eyk

Elucidating the central role of protein phosphorylation in the regulation of muscle contraction on both beat-to-beat and long-term bases began with a series of classical studies starting in the mid-1970s. Despite years of extensive studies, two articles in this issue of Circulation Research demonstrate that there is still a great deal to be learned about the complexity of the myocyte and its regulation by kinases.

Protein kinase A (PKA) has been studied using purified proteins, isolated subproteomes, isolated myocytes, intact heart preparations, and in vivo animal studies, and yet we still do not have the whole picture. The work presented by Chu et al provides a tantalizing glimpse into widespread protein changes occurring in isolated myocytes with isoproterenol treatment. Although phosphorylation of the regulatory proteins phospholamban and cardiac troponin I (cTnI) under β-adrenergic stimulation has been well-characterized, it is apparent that many more proteins are phosphorylated. How could we have missed these other proteins in previous work?

In contrast, Ke et al focus on the role of p21-activated kinase (PAK), a Ser/Thr kinase that is a relative newcomer to muscle research, and demonstrate that overexpression of the active form of PAK1 in isolated myocytes alters cardiac contractility through phosphorylation of the phosphatase, PP2A. Previous work using a variety of PAK isoforms has illustrated the ability of this kinase family to alter smooth and cardiac muscle contraction, but an incomplete molecular picture has been provided. Why were some protein alterations missed in previous work, and how can the results be melded to provide a deeper understanding of the biology?

In both cases, the full range of protein alterations was lost in part due to the experimental approach used to identify phospho-proteins. The key difference in these two new pieces of work lies in the use of a broader-based approach as opposed to a focused approach.

Two approaches are most widely used to determine phospho-targets: (1) addition of a purified kinase to a purified protein or specific subproteome (such as isolated myofibrils) or (2) overexpression of the active kinase or upstream activation of the kinase (or kinase cascade) with a known activator in isolated cells. In all cases, it is assumed that the phospho-proteins identified will be the same as the in vivo targets. But, is this a valid assumption? Certainly, PAK phosphorylates different proteins depending on which protein system is used. For example, purified cTnI can be phosphorylated in vitro by PAK1 and PAK3, but it was not phosphorylated (or had reduced phosphorylation) in more "structurally realistic" systems such as skinned muscle fibers or cardiomyocytes. Three reasons may be proposed for these differences. First, there could be PAK isoform specificity differences (ie, PAK1 versus PAK3). However, the active catalytic domains of PAK1 and PAK3 are highly conserved (>90%), suggesting (but not necessitating) highly similar substrate specificity. Second, phosphorylation may depend on the structural environment—whether cTnI is isolated, part of the trimeric troponin complex, or part of an intact thin filament. Each form of TnI exhibits different conformations depending on its environment and therefore may have structurally “hidden” phospho-epitopes—an effect that is known to occur with other kinases. Third, activation of a phosphatase (ie, PP2A) could remove the PAK-induced phosphorylation of other targets. Thus, less complex protein preparations illustrate the potential of the kinase action, but this case demonstrates that the full picture of the kinase activity is sometimes only seen with broader analysis.

There are many practical reasons for using simplified protein systems. One of the most important is the ability to directly link cause and effect. With a small number of proteins, a change in the function of an individual protein or subproteome can be directly attributed to a specific protein alteration(s). While this is true for the whole myocyte, it is infinitely more difficult to infer a direct link between a change in the overall cellular phenotype and any given protein change, because all of the subproteomes that comprise the myocyte collectively dictate the cellular phenotype. The extensive functional interplay between the various subproteomes means that even a single protein change to one subproteome can result in compensatory alterations to many other subproteomes. For example, a change in force production can be due to changes in the myofilaments and/or calcium loading of the sarcoplasmic reticulum (SR). As such, a detrimental protein change in the myofilament subproteome could be compensated for by a protein change that enhances SR calcium or release. The cellular phenotype under these conditions is thus determined by the ratio of the detrimental to compensatory changes. The intact heart is even more complex, being composed of not just many subproteomes, but many different cell types, which all contribute to the overall function of the organ.

Simpler (or reductionist) protein/experimental systems offer simpler and potentially more straightforward answers. In addition, the minimalist approach reduces confounding fac-

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From the Division of Cardiology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Md.

Correspondence to Jennifer Van Eyk, Johns Hopkins University, Bayview Proteomics Center, Departments of Medicine (Cardiology), Biological Chemistry and Biomedical Engineering, 602 Mason F. Lord Building, Center Tower, Bayview Campus, Johns Hopkins University, Baltimore, MD 21224. E-mail jvaneyk1@jhmi.edu

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tors (such as subcellular localization), provides sufficient protein for in-depth characterization (since identification of the phospho-amino acid often requires micrograms to milligrams of purified protein, even with the most sophisticated techniques), and allows measurement of specific functional characteristics that cannot be measured in vivo due to interplay between subproteomes. Yet, the loss of potentially important protein changes makes one question the need to also look broader at the cell and the heart.

Proteomics is a scientific approach that attempts to view the proteome (or particular subproteome) in a relatively unbiased manner. It is not possible with the current analytical protein methods to view the complete proteome, although techniques like 2D gel electrophoresis (2DGE) afford observation of hundreds to thousands of proteins at a given time. In Chu et al, isolated cardiomyocytes were stimulated with isoproterenol to activate PKA, and analyzed by 2DGE, by separating the whole cell lysate across a single pH gradient. This particular analysis scans only the most abundant cardiac myocyte proteins, with key low abundance and hydrophobic proteins not observed, including most of the SR proteins. Even so, this work clearly demonstrates that isoproterenol treatment results in the phosphorylation of many cellular proteins, illustrating the widespread changes that occur throughout the cell.

An unexpected finding was the phosphorylation of heat shock protein, p20 (HSP20), with isoproterenol treatment. Heat shock proteins are molecular chaperones that play a role in cellular protection. p20 is present in all three muscle types and can be phosphorylated under different conditions, including insulin stimulation of skeletal muscle, and with cGMP-linked vasodilators (such as nitroglycerin) in smooth muscle. It is an exciting discovery to link p20 alterations with the general muscle response/protection system?

One special aspect of the work of Chu et al is that the authors carried this initial discovery all the way through to the physiology and showed that expression of p20 alters contractility. The mechanism of action is not clear, although there are some clues existing in the literature. p20 is known to be part of a large myofibrillar complex in skeletal muscle containing the phosphatase PP1C and other HSPs, including αB-crystallin (to which it shares a high degree of sequence homology), and has been shown to modulate smooth muscle relaxation, increase cardiac muscle shortening, and translocate to the nucleus. Whether PKA activation results in or modifies one or all of these cellular responses is not clear, but these authors have the tools for future investigations.

A particularly interesting aspect of this work, which raises many important issues about identification and naming of proteins, is determining whether the protein observed is simply p20, a unique cardiac homologue of p20, or a novel protein. The ambiguity arises from the mass spectrometry methods that are now commonly used to characterize and identify proteins. Protein spots are isolated from 2D gels and digested with trypsin, which cleaves proteins primarily after arginine and lysine residues, producing a specific set of peptide fragments, known as a fingerprint. The masses of some, but not all, of these fragments are determined using mass spectrometry. The observed mass spectrum, or peptide fingerprint, can be compared with the theoretical spectra of proteins present in protein databases, known as in silico tryptic digests. Protein identification is therefore based on the fit between the observed and theoretical spectrums. In addition, tandem mass spectrometry (MS/MS) can be used to further fragment one or more tryptic fragments yielding amino acid sequence information. The success of this approach is tightly tied to the quality and extensiveness of the protein databases and is ultimately dependent on having the amino acid sequence for every cardiac protein for every species. In the case of Chu et al, the identification of cardiac p20 is based on homology between the known amino acid sequences of p20 from other species and other tissue types. Therefore, even after cloning of p20 from cardiac muscle, differences in the amino acid sequence can be attributed to either species or isoform differences.

Both of these works add to the broader picture of kinase control in cardiac muscle. It is well recognized that the phosphorylation status of a protein is determined by the activity of the kinase and phosphatase. Under conditions of Ke et al, the phosphatase activity appears to dominate. Thus, one can speculate that PAK autoregulates its effect on cTnI and any other protein target by directly controlling PP2A activity through phosphorylation. This also suggests that the functional status of cTnI (and other myofilament proteins) is so critical for cardiac function that the direct effect of PAK on TnI (phosphorylation of Ser149 [at least by PAK3]) is tightly controlled though a feedback mechanism ultimately regulated by PAK itself, which is a very interesting prospect. Likewise, the ability of p20 to alter contractility opens the door to important questions about the role of p20 in cardiac physiology and pathophysiology. As this and other groups probe deeper into the myocyte proteome, other unexpected protein involvements will be uncovered.

In conclusion, Ke et al and Chu el al have illustrated the true complexity of kinase action. The use of broad-based experimental approaches allowed these groups to delve beyond the surface of these processes. In the future, combining focused and broader-based experimentation will allow, in a manner similar to the use of coarse and fine-tuning on a microscope, to zoom in and out of different aspects of the myocyte.

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References


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