Identification of Novel Signaling Complexes by Functional Proteomics

Peipei Ping

Abstract—The rapid development of proteomic technologies, combined with the completion of the Human Genome Map, has enabled the compiling of an unprecedented inventory of cellular proteins. Functional proteomics is an emerging field that aims to utilize the enormous amount of information provided by these proteomic technologies to understand the functions of cellular proteins. The utility of functional proteomics has been recently exploited to elucidate cellular mechanisms in numerous fields, of particular salience in the area of signal transduction. This review presents a functional proteomic approach for the study of cardiac cell signaling. It illustrates the strategies by which the subproteome of a targeted signaling system is characterized in an unbiased fashion, the manner in which the biochemical functions of this subproteome are assessed using established molecular and protein chemistry methods, and the challenges associated with these studies. (Circ Res. 2003;93:595-603.)

Key Words: functional subproteomes ■ multiprotein complexes ■ signal transduction ■ ischemic injury ■ cardiac cell death
Functional Proteomics: Strategies For Hypothesis-Driven Research

Figure 1. Schematic representation of the functional proteomic strategy for hypothesis-driven research. If one starts with a hypothesis, the analysis of protein complexes (eg, gel filtration) provides candidate proteins involved in the process of interest. These candidate proteins are then validated by classical biochemical and physiological techniques, which characterize the role (if any) of this protein in the signaling system responsible for the phenotype.

Applications of Functional Proteomics

Although functional proteomics has been used to elucidate cellular mechanisms in numerous fields, a particular area shown fruition is signal transduction.7–15 Soskic et al14 examined the subproteome containing proteins for the platelet-derived growth factor (PDGF) receptor signaling pathways. They tested the hypothesis that altered phosphorylation events, which occur in an array of proteins, are indicative of molecules that participate in PDGF signaling. Their results showed that PDGF stimulation significantly impacted the serine and tyrosine phosphorylation of proteins within the PDGF receptor signaling system. A similar approach was taken to study N-methyl-D-aspartate (NMDA) receptor signaling in the brain.11 Husi et al11 was the first to report that proteins mediate the NMDA receptor signaling are physically associated with the receptor. The NMDA multigene protein complexes may bear great biological significance during the regulation of neuronal synaptic activity. To understand the role of oxygen tension in placental development, Hoang and colleagues10 determined the effects of hypoxia on the cytotrophoblast proteome. In this study, a subproteome was identified that consisted of proteins that were altered by oxidative stress. This subproteome was found to contain proteins involved in glycolysis, stress response, as well as signal transduction.10 Using an ensemble of techniques similar to those described above, mitogen-activated protein kinase (MAPK) cascades were also characterized by other investigators.13 In yeast, Cdc5p, Cef1p, and hCDC5 were previously identified in the process of pre-mRNA splicing and are components of multiprotein complexes. Proteins stably associated with the multiprotein Cdc5p complexes in both fission and budding yeasts were characterized using tandem affinity purification.15 This work marks the first comprehensive analysis of CDC5-associated proteins in yeast and provides evidence that the members of this complex must cooperate in certain aspects of pre-mRNA processing.

A unique feature of these functional proteomic studies is the step taken beyond the identification of proteins within a subproteome, that is, to characterize the functions of the identified proteins (Figure 1). For example, in the report on PDGF receptor signaling,14 the proteins identified by mass spectrometry were further studied to understand their roles in the interaction between multiple signaling pathways, and to examine kinetic changes in the posttranslational modifications after PDGF stimulation. In the investigation of cytotrophoblast proteome,10 the subcellular location of identified proteins was further determined using confocal microscopy to characterize the redistribution of these proteins in the wake of hypoxia. In this regard, functional proteomic analyses serve to reveal the functions of proteins and to couple these functions with the cellular mechanisms responsible for cell and organ phenotypes.

Using Functional Proteomics to Map Multiprotein Signaling Complexes

The Signaling Module Hypothesis

Despite the unprecedented advances in the discovery of novel signaling elements in recent years,16,17 little is known regard-
ing the specific manner in which these molecules interact with each other and by what mechanisms they transduce signals. Essentially, the blueprint for the cellular signaling architecture remains to be unmasked.

Subcellular signaling has been classically defined as a host of linear pathways, in which molecule X is located either upstream or downstream of molecule Y, and the summation of many Xs and Ys in turn constitutes a system that transduces a signal. This linear paradigm of signal transduction, although simple, has provided satisfactory guidance for the obscured cell signaling mechanisms over several decades. However, the validity of this model is being frequently challenged by many recent observations that suggest signal transduction is a decidedly more dynamic process. Specifically, it has lately been reported that signaling systems appear to accomplish a multitude of distinct tasks with a limited set of proteins,\textsuperscript{16,17} i.e., the cellular phenotypes A and B may be dramatically different, but the molecular participants in the generation of these phenotypes may be similar or even identical. Signaling by the mitogen-activated protein kinase (MAPK) superfamily in cardiac cells represents an intriguing exemplar of this phenomenon.\textsuperscript{8,18–21} Cardiac cell hypertrophy has been shown to involve differential activation of ERK and JNK.\textsuperscript{19,20} In addition, both ERK and JNK kinases have been demonstrated to participate in the signaling responses of an entirely different phenotype: cardioprotection against ischemic injury.\textsuperscript{8,21} The resultant questions are as follows: How does the cell recruit identical or suitably expressed signaling proteins to achieve task-specific signal transduction? How does the individual multiprotein protein complex can be purified against one of the components. This method isolates the individual complexes. It is plausible that by regulating the protein components of various multiprotein complexes, a cell would be able to conduct signal transduction to manifest a wide spectrum of phenotypes.

### Functional Proteomic Strategy to Characterize a Signaling Complex

#### Profiling a Signaling Complex

Several methodologies have been utilized to isolate protein complexes.\textsuperscript{7,8,25,26} Often, the first step is to obtain a concentrated subcellular fraction of interest.\textsuperscript{53–58} From this fraction, the individual multiprotein protein complex can be purified via a series of non-denaturing techniques.\textsuperscript{15,57–60} The goal of these studies is to maintain (1) the associating members of a protein complex and (2) their molecular interactions with each other, because these features exist endogenously. The characterization of PKC\textsubscript{ɛ} multiprotein complexes in cardiac mitochondria is given as example of this approach (Figure 2). The approach will garner information about the molecular composition of the complex, moreover, about the native size and molecular weight of signaling complexes.

Multiprotein complexes may be isolated and purified by a variety of methods, among which, antibody-based immunoprecipitation and recombinant protein-based affinity pull-down are most effective. Importantly, these two techniques provide complimentary information about protein complexes when used in parallel. Antibody-based immunoprecipitation targets preexisting multiprotein complexes using an antibody against one of the components. This method isolates the complexes without disrupting them, but is limited in its ability to identify specific complexes in which the antibody-directed epitope may be masked by other interacting proteins. Additionally, the antibody-binding event may displace proteins that interact with the epitope region of the target protein, and hence these interacting proteins would escape the analysis as well. In contrast, recombinant protein-based affinity pull-down assays introduce exogenous full-length proteins, i.e., the “bait” protein, into the system. In this assay, the bait protein competes with endogenous protein to form a new multiprotein complex. This method is advantageous because epitope tagging of the recombinant protein facilitates the pull-down and reduces the likelihood that the epitope for
isolation is masked. When used together, these two tools are a powerful approach to isolate and purify multiprotein complexes. In addition, liquid chromatography and other non-denaturing tools (eg, blue native electrophoresis) can be used in combination with antibody-based immunoprecipitation and recombinant tag protein based affinity pull-downs to enhance the sensitivity of the purification procedure (Figure 2). At this stage, the multiprotein complexes can also be visualized by electron microscopy after isolation to gain knowledge on the physical properties of the complexes.\textsuperscript{41,42,44}

Subsequently, the proteins in the complexes can be further separated by standard denaturing electrophoresis and identified by either matrix-assisted laser deionization (MALDI) mass spectrometry or tandem mass spectrometry.\textsuperscript{3,4,45} Based on the experience we have obtained in characterizing PKC\textsubscript{e} multiprotein complexes in the mitochondria, successful identification of molecular components within these complexes requires a high-sensitivity approach. Often, low-flow rate electrospray (“nanospray”) with reverse-phase LC and tandem MS on gel excised and trypsinized proteins are effective.\textsuperscript{4,7,43} This approach has high sensitivity and mass accuracy, it generates primary amino acid sequence data, and increases the likelihood of definitive identification of proteins in the subsequent database search. LC/MS/MS has been implemented to determine components of the splicesome multiprotein complex\textsuperscript{43} and the NMDA receptor complex.\textsuperscript{11}

\textbf{Figure 2.} Example application of a functional proteomic strategy to examine PKC\textsubscript{e} multiprotein signaling complexes. Cardiac mitochondria are isolated and their functional viability confirmed. Next, mitochondrial lysates are incubated with recombinant GST-tagged PKC\textsubscript{e} protein, and GST-based affinity pull-down assays are performed to purify PKC\textsubscript{e} protein complexes. These purified protein complexes are separated by blue native polyacrylamide gel electrophoresis (PAGE), a non-denaturing separation technique developed for the mitochondria that does not disrupt protein-protein interactions within the complexes. Parallel purification experiments are conducted using the GST epitope alone (ie, GST-null proteins incubated with mitochondria) and solely the GST-PKC\textsubscript{e} protein (without mitochondrial proteins) to serve as negative controls for the blue native PAGE step. Thus, after blue native PAGE, specific protein complexes isolated via GST-PKC\textsubscript{e} pull-down are excised. These multiprotein complexes are in-gel digested and identified by liquid chromatography–coupled tandem mass spectrometry analysis (ionization by electrospray). Lastly, the specific suborganellar distribution of the identified proteins across various compartments of the mitochondria are determined by fractionation of the mitochondria into outer membrane, intermembrane space, inner membrane, and matrix components, followed by immunoprecipitation for PKC\textsubscript{e} and immunoblotting with antibodies corresponding to identified proteins.
and to examine multiprotein complexes in yeast when coupled with affinity pull-down and PAGE separation.\(^\text{25,26}\) After mass spectrometry analysis, the protein sequence data are searched against the protein sequence database (such as the NCBI non-redundant database), using one of numerous publicly available search engines.\(^\text{4,7,45}\)

**Characterization of Signaling Modules**

There are four important features of a signaling module that delineate the strategies by which it is characterized. First, a module consists of a set of proteins that are physically associated with each other. The identities of proteins in a module can be determined via mass spectrometry or immunoblotting; whereas their physical interactions are assessed via either in vitro affinity pull-down assays and/or communoprecipitation. Second, the assembly of a module usually leads to chemical modification (i.e., posttranslational modification) of one or more of the module components, which can be determined via mass spectrometry and/or 2-dimensional electrophoresis. Third, a signaling module is a basic functional unit within the complex, and thus its ability to transduce signals from one molecule to the next is analyzed using biochemical assays (e.g., kinase activity assays). Finally, a signaling module consists of those members of a complex that necessarily associate in order to carry out a signaling event, and hence the functional significance of modules must be verified by the phenotype that they engender. The necessary role of a given module in the signaling system underlying a phenotype is often assessed by pharmacological studies and the use of transgenic animals.\(^\text{27,28}\) For example, the establishment of a hypertrophic signaling module will require studies that involve the disruption of this module’s assembly in vivo and the examination of its impact on cardiac hypertrophy.

**Identification of Binding Partners**

A number of techniques have been utilized to identify binding partners of a protein.\(^\text{27,28,46–49}\) Often the protein of interest is used as a bait to screen for candidate binding partners via affinity pull-down assays. An alternative method that has been widely exploited is the yeast two-hybrid screen,\(^\text{49}\) whereby a reporter gene is activated as the result of interactions between the bait and “prey” proteins. The yeast two-hybrid assay was recently used in a high-throughput fashion to delineate the protein-protein interaction map of *Helicobacter pylori*.\(^\text{39}\) Once two proteins have been characterized as binding partners, the molecular interactions between various domains of these molecules of interest can subsequently be deciphered using mutagenesis studies.\(^\text{27,30}\) It is noteworthy that most of the technologies discussed herein will identify a large pool of proteins that are able to interact with a protein of interest. However, these findings are not necessarily indicative of what occurs in a living cell. Therefore, it is essential that the putative binding partners identified in vitro are further validated with in vivo experiments (Figure 1).

**Mechanisms That Define Complex Formation and Module Assembly**

Although a number of reports have documented the existence of multiprotein complexes in a variety of systems,\(^\text{7,8,11,15,22–26}\) little is known regarding the cellular mechanisms that regulate the assembly of these complexes. Current investigations from our laboratory and others suggest that subcellular location, cell stimulus, and the molecular configuration of proteins may contribute to complex assembly and module formation.\(^\text{5,8,21,27,28,38}\)

Evidence indicating that protein complexes may be compartmentalized at targeted subcellular locations has come from several investigations.\(^\text{16,17,35,36,50}\) In particular, characterization of PKCe complexes from total cardiac cell lysates and analyses of MAPK complexes from the K562 cells both identified a group of organelle-specific structural proteins that associate with these kinases. Specifically, PKCe was found to associate with caveolin-3 and prohibitin, whereas the MAPKs were colocalized with the Golgi reassembly stacking protein and the nuclear transport RNA-binding protein, suggesting that these complexes might be differentially sequestered to various subcellular compartments.\(^\text{12}\) Further analysis of the PKCe complexes showed that the composition of all PKCe complexes was not identical across different cellular compartments. Instead of a singular PKCe complex that simultaneously includes all PKCe-associated molecules, each subcellular compartment appears to posses a unique PKCe complex, the composition of which appears to be distinctly tailored to perform the functions of the specific subcellular organelle (Figure 2).\(^\text{38}\) For example, a cardiac module containing PKCe and Src tyrosine kinase formed only in the particulate regions of the cell. Although PKCe and Src were both independently found in the cytosolic fraction, PKCe-Src modules were not detected in this subcellular location of the cardiac cell.\(^\text{28}\) Taken together, these findings are consistent with the signaling module hypothesis that subcellular location modulates the assembly of complexes and modules.

Stimulus-dependent regulation of a subproteome has recently been demonstrated in multiple cell types.\(^\text{10,12,14,51,52}\) For example, the phosphorylation events in the PDGF receptor signaling subproteome were significantly affected by PDGF stimulation.\(^\text{14}\) In the human K562 cell proteome, phorbol esters dynamically impacted the posttranslational modifications of MAPK cascade members.\(^\text{12}\) These data illustrate that a stimulus (such as PDGF and phorbol esters) can profoundly influence the assembly of protein complexes, both by modulating the composition of the complex and by governing the biochemical characteristics of molecules associated with the complex. Recent data from our laboratory demonstrated that formation of PKCe-Lck modules was significantly increased during two forms of cardioprotection: ischemic preconditioning and PKCe-induced protection.\(^\text{27}\) indicating that the impinging stimulus also appears to heavily regulate module formation.

For most cellular kinases, subcellular translocation and activation is accompanied by molecular conformational changes.\(^\text{16,47}\) The manner by which the configuration of a molecule influences the formation of signaling modules was established through mutation-based in vitro analyses of PKCe and Src tyrosine kinase.\(^\text{30}\) Additional data from our laboratory indicates that Src tyrosine kinase favors binding to the open configuration of PKCe, whereas its interactions with the
wild-type PKCɛ were considerably weaker.\textsuperscript{30} It has been well documented that the particulate PKCɛ protein exhibits an open conformation, whereas in the cytosol, the kinase assumes a closed configuration. Thus, the finding that PKCɛ-Src modules reside exclusively in the particulate fraction and that the binding of Src and PKCɛ is enhanced when PKCɛ is in an open configuration, underscores the notion that subcellular location and molecular configuration play essential roles in module assembly.

Complex Formation as a Mean for Cardiac Cell Signaling
What are the advantages of conceptualizing cardiac cell signaling as multiprotein complexes? Cell signaling via complex formation affects the tenets of the current signaling paradigm in at least three ways. First, this model focuses on not only the central kinase/molecule orchestrating the signaling event, but importantly, it also takes into account the battery of molecules that function in concert to accomplish signal transduction. In this regard, emphasis is placed on both the associating partners of the central kinase/molecule as well as the specific manner by which these partners interact. Second, spatial distribution of these multiprotein complexes appears to contribute to the dynamics of this model. Information obtained by our group\textsuperscript{21,27–29,38} and others,\textsuperscript{35–37,39} although limited, indicates that the subcellular location of the complex is a major determinant of its molecular composition and the task that the complex performs. Third, as many signaling molecules are being explored as potential drug targets, the signaling module hypothesis highlights novel strategies for drug design: agents that target protein-protein interactions, or moreover, that influence the assembly of specific complexes, are more appealing than drugs that aim at a single molecule in isolation. If multiprotein complexes are essential mechanisms of cell signaling underlying phenotype in health and disease, then therapeutic agents designed to effectively promote or disrupt their formation would likely achieve a level of specificity and efficacy not possible with present strategies.

Lessons Learned Through the Characterization of PKCɛ Multiprotein Complexes
Despite the enormous promise functional proteomic strategies afford for the identification of novel multiprotein signaling complexes, a number of limitations associated with the technology also present tremendous challenges for its implementation. Based on our experience with the characterization of PKCɛ multiprotein complexes, several issues are worthy of careful consideration.

Identification of Members Residing in the Multiprotein Complex
In contrast to what had been anticipated in the early stages of functional proteomic studies, the rate-limiting step in this approach is not mass spectrometry–based protein identification (although it is an exceptional technically demanding step), rather, it is the functional validation of proteins identified by mass spectrometry. Our experience with characterizing PKCɛ multiprotein complexes in cardioprotection\textsuperscript{7,8} illustrates, in part, the challenges inherent with these investigations, and the specific measures that may be applied to overcome these obstacles.

The story begins with the PKC hypothesis in preconditioning introduced by Downey and colleagues.\textsuperscript{53} In their 1994 report, inhibition of PKC was shown to block ischemic preconditioning, and activation of this kinase produced an infarct-sparing effect. Subsequently, mounting evidence has demonstrated that the ɛ isoform of PKC is a critical mediator of preconditioning.\textsuperscript{54–59} In addition to the heart, activation of PKCɛ appears to be a conserved signaling step in protection against cell death in a number of organs.\textsuperscript{60–62} Furthermore, multiple ancillary studies have characterized an array of molecules that participate in preconditioning in a PKC-dependent fashion (see review\textsuperscript{54,55}). The data suggest that during the genesis of cardioprotection, PKCɛ does not function in isolation. Rather, PKCɛ appears to form alliances with a battery of other proteins. These findings support a cardioprotective signaling system that is reliant on PKCɛ, but in addition, that includes a repertoire of molecules whose participation is tightly coupled to the activation of PKCɛ. This enigma of PKCɛ signaling provided an ideal opportunity for the implementation of functional proteomics to characterize a cardioprotective subproteome.

Using a functional proteomic approach, we isolated and purified the PKCɛ multiprotein complexes (an example of such is shown in Figure 2). Although performing the appropriate negative controls for the immunoprecipitation (eg, IgG-based immunoprecipitation as negative controls\textsuperscript{7,8} or the affinity pull-downs (eg, GST-null protein as negative controls\textsuperscript{7,8}; Figure 2) significantly reduced the number of false-positives, the most effective measure was the implementation of criteria, on the basis of which we could discern the hundreds of gel plugs collected from either 1-dimensional or 2-dimensional gel electrophoreses and subsequently analyzed using both MALDI mass spectrometry and LC tandem mass spectrometry. We reasoned that a protein must satisfy the following prerequisites to be considered as a true candidate: (1) identification of the protein was confirmed in multiprotein complexes purified by two independent approaches (eg, immunoprecipitation and GST-affinity pull-down); (2) the protein must have been identified by mass spectrometry on the basis of at least two peptides; and (3) its association with PKCɛ verified by subsequent immunoprecipitation and immunoblotting studies. Finally, studies demonstrate that the association with PKCɛ complexes result in the modification of the activity and/or biological function of a molecule are taken as solid evidence to support a functional role of such molecule in the PKCɛ complexes. With this set of criteria, a total of 93 proteins (from several hundred analyzed spots) have been identified as candidate members of various PKCɛ multiprotein complexes in the heart.\textsuperscript{7,8}

Functional Characterization of Modules
Based on data in the literature, these 93 proteins can be divided into at least 6 functional groups and are known to localize to various subcellular locations. Our studies show
that these components were dynamically altered during cardioprotection. Thus, the task of target validation continues at the level of establishing a causative relationship between a particular signaling module and the genesis of a specific phenotype. To this end, we have thus far characterized the PKCe-Src tyrosine kinase signaling modules, the PKCe-Lck signaling modules, and the PKCe-MAPK signaling modules and PKCe-VDAC signaling modules at the mitochondria. Interestingly, the data suggest that although each modules contains PKCe, the downstream signaling events that these modules regulate appear to be distinct. For example, our studies demonstrate that an important downstream signaling task of the PKCe-Lck module is the mobilization of the transcription factor NF-κB, a molecule that had been previously shown to play a protective role by several other investigators (see review) although its mechanism of activation in cardioprotection was not understood. In another example, a signaling target of the mitochondrial PKCe-ERK modules was found to be the proapoptotic protein Bad, phosphorylation and inhibition of which was observed in the protected myocardium.

The characterization of PKCe signaling complexes and modules not only aided the verification of protective molecules suggested by previous studies, but more importantly, it has led to the identification of novel signaling modules that had not been discovered by classical approaches. Two examples in this regard are the classification of PKCe-Bmx and the PKCe-Oracle/Cypher signaling modules within the PKCe multiprotein complexes. Both proteins were previously unrecognized as potentially participating in cardioprotective signaling. Bmx and Oracle/Cypher are identified by MALDI-MS (Bmx) and LC/MS/MS (Oracle/Cypher) as members of the PKCe multiprotein complexes. In view of these lines of evidence, it is conceivable that multiple distinct modules within the PKCe complex may exist. These modules function to facilitate the genesis of the cardioprotective phenotype by the accomplishment of as yet unknown signaling tasks in targeted subcellular locations.

Challenges Ahead

Through distinct applications in a variety of studies, functional proteomics has proven to be a powerful tool for the identification of novel multiprotein complexes, signaling modules, and binding partners. Nevertheless, the current technology has several limitations, including the following: (1) it identifies stably associated multiprotein complexes and may not faithfully recapitulate all protein-protein interactions present in vivo; thus, a protein that transiently associates with a multiprotein complex may escape the identification; (2) the current approach is not robustly quantitative and does not provide information regarding the stoichiometry of any components within the multiprotein complex; (3) the sensitivity is low and may not identify proteins that are less abundant; and (4) only those proteins whose interaction are not affected by the given affinity purification procedure can be identified. The last issue may have significant impact, because the purification procedure may affect posttranslational modifications and protein-protein interactions. Ostensibly, efforts to address these issues are much needed and are necessary steps to make this approach more effective.

Concluding Remarks

As is the case with the genome, cellular or whole animal proteomes will not in themselves represent usable ends. If the tremendous amount of proteomic data are instead taken in the light of a specific hypothesis and used to understand the signaling architecture that is responsible for physiological and pathophysiological organ condition, then the true power of proteomics can achieve fruition. In this regard, functional proteomics affords a nonbiased investigation of a subproteome of interest, along with subsequent examination of this subproteome on the basis of a specific hypothesis to interrogate specific protein events that manifest cell or organ phenotypes. Importantly, functional proteomics allows not only the identification of novel proteins, signaling modules, and signaling complexes, but moreover, it aids our understanding of the causative relationship between the modification of these proteins and development of pathological organ conditions. It is with this end in hand that we can hope to see the conceptual elegance of molecular medicine become a reality.

Acknowledgments

This work was supported in part by NIH R01 grants HL63901 and HL65431 and by the Laubisch Endowment. The author is deeply indebted to Dr Thomas M. Vondriska, who has provided essential discussions and critical comments on this review.

References


Identification of Novel Signaling Complexes by Functional Proteomics

Peipei Ping

Circ Res. 2003;93:595-603
doi: 10.1161/01.RES.0000093221.98213.E0

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/93/7/595

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the
Editorial Office. Once the online version of the published article for which permission is being requested is
located, click Request Permissions in the middle column of the Web page under Services. Further information
about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/