

Functional Proteomic Analysis of Protein Kinase C ϵ Signaling Complexes in the Normal Heart and During Cardioprotection

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Abstract—Using two-dimensional electrophoresis, mass spectrometry, immunoblotting, and affinity pull-down assays, we found that myocardial protein kinase C ϵ (PKC ϵ) is physically associated with at least 36 known proteins that are organized into structural proteins, signaling molecules, and stress-responsive proteins. Furthermore, we found that the cardioprotection induced by activation of PKC ϵ is coupled with dynamic modulation and recruitment of PKC ϵ -associated proteins. The results suggest heretofore-unrecognized functions of PKC ϵ and provide an integrated framework for the understanding of PKC ϵ -dependent signaling architecture and cardioprotection. (*Circ Res.* 2001;88:59-62.)

Key Words: protein kinase C ϵ ■ stress-activated kinases ■ stress-activated proteins

The ϵ isoform of protein kinase C (PKC ϵ) has recently become the focus of considerable interest because of its critical role in protecting the myocardium against ischemia/reperfusion injury.¹⁻⁴ Cogent evidence indicates that the activation of PKC ϵ is a pivotal event in the development of the cardioprotective effects of ischemic preconditioning.¹⁻⁴ Moreover, transgenic overexpression of low levels of active PKC ϵ ^{2,3} or of a peptide that causes its activation⁴ results in a cardiac phenotype characterized by enhanced resistance to myocardial ischemia/reperfusion injury, ie, a phenotype analogous to that observed during preconditioning. Thus, recruitment of PKC ϵ is both necessary and sufficient to confer cardioprotection, emphasizing the unique role of this isozyme in the pathophysiology of myocardial ischemia. However, the precise molecular signaling mechanisms underlying PKC ϵ -dependent cardioprotection remain unknown.

In the complex molecular infrastructure that underlies preconditioning, PKC ϵ appears to be an upstream element, orchestrating a series of signaling events that result in the recruitment of many of the downstream factors (kinases, transcription factors, and other proteins) involved in the acquisition of cardioprotection.¹⁻⁴ Thus, identification of the molecules that participate in PKC ϵ signaling may provide important insights into the molecular basis of both preconditioning and cardioprotection. In noncardiac cells, it is becoming apparent that signaling molecules operate in close proximity,⁵ forming complexes that aid the transmission of signals.⁵ However, virtually nothing is known regarding whether PKC ϵ forms signaling complexes in the heart, and, if

so, which specific proteins participate in PKC ϵ -dependent signaling complexes. Elucidation of these issues is an indispensable first step toward unraveling the mechanism of PKC ϵ -dependent cardioprotection.

Thus far, studies of signaling in preconditioning have adopted a conventional approach, in which the investigation is focused on one or few target kinase(s).¹⁻⁴ Although this narrow approach has provided many important insights into the mechanism of preconditioning, it is inherently limited in its ability to offer a comprehensive and systematic analysis of the multiple signaling events that underlie this phenomenon. Although individual molecules may trigger important signaling responses, it is the coordinated interaction of multiple kinases within a signaling complex and the subsequent integrated action of multiple complexes that bring about the manifestation of a phenotype. In recent years, the development of proteomic technology has made it possible to examine multiple proteins and their interactions on a large scale.⁶ Two-dimensional (2D) gel electrophoresis coupled with advanced mass spectrometry allows high-throughput, systematic display of a complete protein profile and comprehensive assessment of multiple molecules in parallel.⁶ Rather than examining a single molecule in isolation, functional proteomic strategies are aimed at obtaining information regarding the expression profile of all proteins as well as the protein interactions within a signaling complex, thereby providing a holistic portrait of the entire signaling network.

Accordingly, in the present study, we adopted a novel functional proteomic approach to gain insights into the PKC ϵ -dependent signaling events that lead to cardioprotection. We specifically sought to determine whether PKC ϵ

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Proteins That Reside in PKC ϵ Complexes

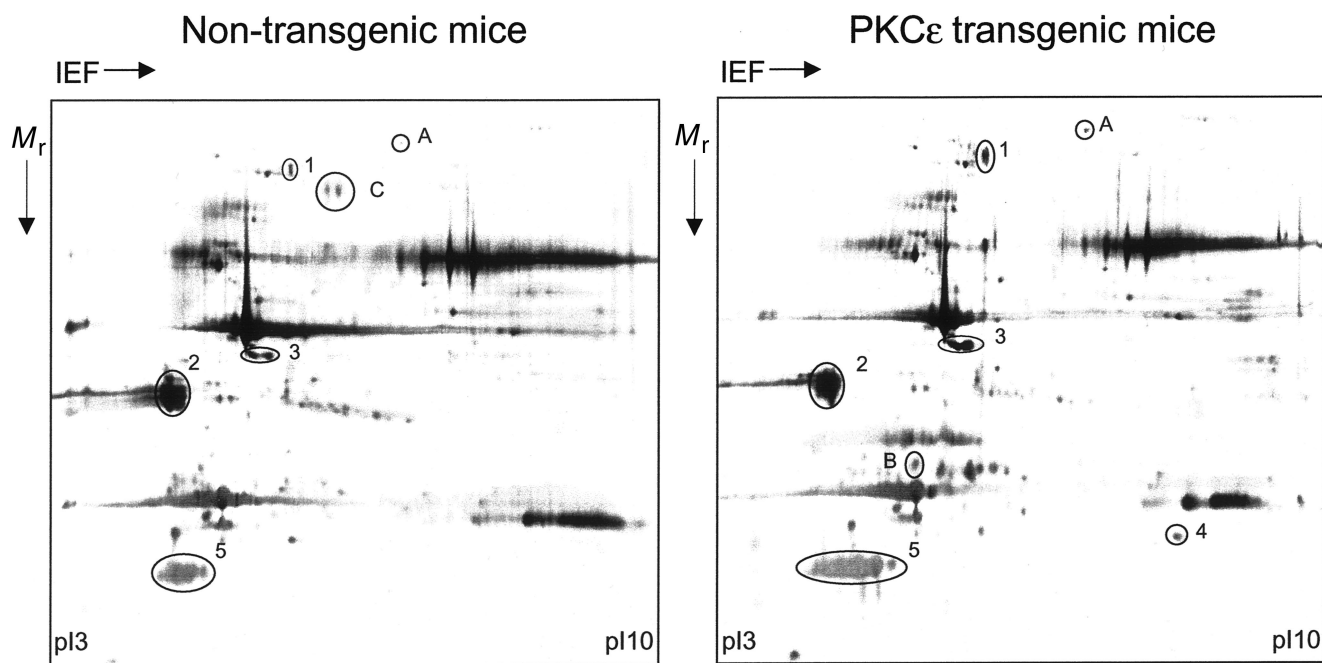
	M_r , kDa	Known Function	Accession No.	Altered PTMs in PKC ϵ Mice	Altered Expression in PKC ϵ Mice
Structural and Skeletal Proteins					
1. Cardiac α -actin	42	Cytoskeletal protein	CAA27398	...	+
2. Troponin T2	35	Contractile protein	NP035749	Yes	++
3. α -Tropomyosin	33	Contractile protein	CAA46043	Yes	+
4. Prohibitin	30	Mitochondrial membrane protein	P24142	...	NC
5. GM130	130	Golgi matrix protein	Q62839	...	++
6. Villin	95	Cytoskeletal protein	Q62486	...	+
7. Lap 2	53	Nuclear membrane protein	NP035735	...	++
8. Desmin	53	Cytoskeletal protein	P31001	Yes	++
9. Caveolin-3	18	Cardiac caveolae protein	NP031643	Yes	+
10. Adaptin- β	106	Adaptor protein	CAA69224	...	+++
11. Myosin light chain	19	Contractile protein (cardiac isoform)	P51667	Yes	+
Signaling Proteins					
12. PI3 kinase	170	Activator of PKC	AAB07682	...	+
13. PI3 kinase	85	Subunit of PI3 p170 kinase	A38747	...	++
14. Connexin43	43	Cardiac gap junction protein	P23242	Yes	+
15. Pyk2 kinase	116	FAK tyrosine kinase family	AAC50203	Yes	+
16. Src tyrosine kinase	60	Src tyrosine kinase family	P05480	Yes	+
17. Lck tyrosine kinase	56	Src tyrosine kinase family	P06240	Yes	+
18. BMX	80	Cytoplasmic tyrosine kinase	AAB47770	Yes	+++
19. PKB α /Akt	59	Stress-activated kinase	P31750	Yes	+++
20. p38 MAPK	38	Stress-activated kinase	P47811	Yes	+
21. MAPKAPK2	42	Stress-activated kinase	P49138	Yes	+
22. JNK1 (p46)	46	Stress-activated kinase	BAA85875	Yes	+++
23. JNK2 (p54)	54	Stress-activated kinase	BAA85876	Yes	+
24. MEK1	45	Activator of ERK pathway	P31938	...	+
25. ERK1 (p44)	44	Extracellular signal-regulated kinase	P21708	Yes	+
26. ERK2 (p42)	42	Extracellular signal-regulated kinase	BAA22620	Yes	+++
27. Ras	21	Signal transduction element	AAD56718		++
Stress-Activated Proteins					
28. iNOS	130	Inducible nitric oxide synthase	P29477	Yes	+
29. eNOS	140	Constitutive nitric oxide synthase	P70313	Yes	++
30. COX-2	70	Prostaglandin H synthase	Q05769	...	+
31. Hif-1 α	120	Hypoxia-inducible factor	Q61221	...	+
32. Heme oxygenase-1	32	Stress-activated protein	P14901	Yes	++
33. Aldose reductase	36	Osmotic stress-activated protein	P45376	Yes	+
34. HSP 70	70	Heat stress-activated protein	AAC84169	Yes	+++
35. HSP 27	27	Heat stress-activated proteine	AAA18335	Yes	++
36. α B-crystallin	23	Molecular chaperone	A39608	Yes	+++

PTMs refer to posttranslational modifications as determined by both 2D and MALDI analyses. All proteins were identified using mouse sequences except that rat sequences were used for GM130 and ERK1, and that the human sequence was used for Pyk2 kinase. + indicates a 1- to 2-fold upregulation of protein abundance compared with nontransgenic mice; ++, a 3- to 5-fold upregulation; and +++, a 6- to 10-fold upregulation. NC indicates no change.

forms signaling complexes in the heart and to identify the components of these complexes. Using this approach, we have thus far identified 36 proteins physically associated with PKC ϵ . Our data show that PKC ϵ signaling complexes are associated with multiple subcellular compartments and that activation of PKC ϵ induces dynamic modulation of the expression profile of these complexes.

Materials and Methods

Total cardiac tissue lysates from either 10 PKC ϵ transgenic mice^{2,3} or 10 age-matched nontransgenic littermates were pooled as one sample. A total of 30 mice (3 samples) in each group were studied. PKC ϵ signaling complexes were identified by coimmunoprecipitation with PKC ϵ monoclonal antibodies (PharMingen), followed by 2D electrophoresis, matrix-assisted laser desorption ionization



Representative 2D gels illustrating proteomic profiling of PKC ϵ complexes from nontransgenic mice (left) and PKC ϵ transgenic mice (right). 2D electrophoresis analysis reveals significant changes in the number of protein spots, protein abundance, and the posttranslational modifications of proteins in immunoprecipitates from PKC ϵ transgenic mice compared with nontransgenic mice. Five known proteins (identified via MALDI mass spectrometry) are given as examples. Protein 1 is heat shock protein 70 (pI 5.5; M_r 70 kDa); 2 is the tropomyosin α chain (pI 4.7; M_r 32.7 kDa); 3 is troponin T2 (pI 5.3; M_r 34.5 kDa); 4 is α B-crystallin (pI 7.3; M_r 20 kDa); and 5 is the cardiac isoform of myosin regulatory light chain (pI 4.7; M_r 18.7 kDa). Additionally, three unidentified proteins are also labeled (proteins A, B, and C). Protein A has an approximate pI of 5.9 to 6.0 and M_r of 85 kDa; B has a pI of 4.9 to 5.1 and M_r of 24 to 26 kDa; C has a pI of 5.6 to 5.7 and M_r of 66 to 68 kDa. Note that protein C is downregulated in PKC ϵ transgenic mice. IEF indicates isoelectric focusing.

(MALDI) mass spectrometry, and Western immunoblotting. Formation of PKC ϵ signaling complexes was then independently confirmed by a GST-PKC ϵ -based affinity pull-down assay.⁶ Only those components whose localization in the PKC ϵ complexes was confirmed with the latter assay are reported in the present study. Adult cardiomyocytes were isolated from 6 PKC ϵ transgenic and 6 nontransgenic hearts to verify the expression of all molecules in this cell type. All experiments were performed using a protocol approved by the Institutional Animal Care and Use Committee. A detailed methodology is provided in the online data supplement (<http://www.circresaha.org>).

Results and Discussion

Expression of Structural Proteins in PKC ϵ Signaling Complexes in Nontransgenic Mice

Proteomic analysis revealed the association of several structural proteins with PKC ϵ signaling complexes (Table, Figure), suggesting that these complexes are widely distributed in multiple subcellular locations. Importantly, besides proteins residing in subcellular structures previously known to be associated with PKC ϵ (Golgi apparatus, caveolae, and contractile filaments), expression profiling also identified nuclear apparatus proteins (Lap 2) and mitochondrial inner membrane proteins (prohibitin). Although previous investigations have indicated perinuclear expression of PKC, our results provide the first evidence that PKC ϵ signaling complexes are associated with the inner mitochondrial and nuclear compartments. To exclude the possibility that the presence of these two structural proteins in PKC ϵ complexes may have been an artifact associated with the immunoprecipitation procedure, we isolated cardiac cell nuclei, mitochondria, and caveolae. PKC ϵ monoclonal antibodies

revealed strong immunosignals from each of these preparations, confirming that PKC ϵ signaling complexes do exist in these subcellular compartments. PKC ϵ complexes were also found to be associated with the cytoskeletal proteins desmin, villin, and adaptin- β (Table). Analysis of isolated cardiomyocytes confirmed the expression of all listed proteins in this cell type (Table). The affiliation of PKC ϵ with nuclear apparatus, inner mitochondrial membrane, and cytoskeletal proteins implies novel targets and previously unrecognized functions for this kinase. Our findings warrant further studies to determine whether PKC ϵ plays a regulatory role in modulating the functions of these proteins and to establish the overall functional significance of the observed protein interactions.

Expression of Signaling and Stress Proteins in PKC ϵ Signaling Complexes

As expected, a large number of signaling molecules claim residence in the PKC ϵ complexes (Table, Figure). The potential downstream targets of PKC ϵ , such as Src and Lck tyrosine kinases and mitogen-activated protein kinases (p38 MAPKs, JNKs, and ERKs), and upstream modulators of PKC ϵ , such as PI3 kinases and their substrate, PKB/Akt, are included in this list. Interestingly, these kinases have also been implicated in preconditioning. Colocalization of these kinases in the PKC ϵ signaling complexes indicates potentially important mechanisms for the transmission and integration of signals during preconditioning.

An intriguing finding is the presence of two isoforms of nitric oxide synthase (NOS) (inducible [iNOS] and constitutive [eNOS]) in the PKC ϵ signaling complexes. In noncardiac tissues, it has been

shown that tyrosine kinases are key regulators of iNOS and that Akt is a direct activator of eNOS. However, although both iNOS and eNOS contain multiple potential PKC phosphorylation sites (16 for mouse iNOS and 22 for mouse eNOS), virtually nothing is known regarding PKC ϵ -dependent posttranslational modifications of NOS. Our observations provide the first evidence that iNOS and eNOS (both of which play a fundamental role in preconditioning) are physically associated with PKC ϵ . Other stress-responsive proteins implicated in preconditioning, such as COX-2, Hif-1 α , heme oxygenase-1, heat shock proteins (HSPs), and aldose reductase, were also found in the PKC ϵ complexes (Table). The coexistence of multiple signaling kinases with these stress proteins undoubtedly facilitates posttranslational regulatory events. Taken together, our data suggest a novel role of PKC ϵ in the modulation of iNOS, eNOS, COX-2, Hif-1 α , heme oxygenase-1, HSPs, and aldose reductase, and lay the groundwork for future in-depth and detailed investigations of the mechanisms responsible for the regulation of these proteins.

PKC ϵ -Mediated Cardioprotection Is Associated With Posttranslational Modification and Recruitment of Proteins Into Its Signaling Complexes

To elucidate the molecular infrastructure that underlies PKC ϵ -mediated cardioprotection, we examined transgenic mice expressing low levels of constitutively active PKC ϵ (PKC ϵ activity: 208 \pm 18% of negative littermates), which exhibit a cardioprotected phenotype (ie, enhanced resistance to myocardial ischemia/reperfusion injury) similar to that conferred by preconditioning. Proteomic analysis revealed that PKC ϵ -mediated cardioprotection was associated with two striking changes in PKC ϵ signaling complexes: (1) posttranslational modification of 24 of the 36 proteins identified and (2) altered protein expression of 35 of the 36 proteins identified (Table, Figure). Posttranslational modification was determined by a shift in pI in the 2D electrophoresis and/or by MALDI analysis.

Among the proteins that underwent posttranslational modification in PKC ϵ transgenic mice, 23 of 24 contain potential PKC phosphorylation sites. Although PKC-dependent posttranslational modifications have been implicated in a number of biological functions, events specifically induced by activation of the ϵ isoform have never been defined. Our study represents the first investigation to identify PKC ϵ -induced posttranslational modifications in the heart.

In addition to these posttranslational changes, the current proteomic analysis reveals that PKC ϵ -mediated cardioprotection is associated with remarkable changes in both the quality and the quantity of the components constituting the PKC ϵ signaling complexes (Table). Specifically, we found that some proteins were recruited into the complexes whereas others were no longer part of them (Figure). Among molecules that were recruited to join these complexes in the heart of PKC ϵ transgenic mice, one group consists of new proteins that are not found in wild-type mice (eg, α B-crystallin [Figure]), whereas the second group is composed of proteins that are found in wild-type mice but are expressed with much greater abundance in PKC ϵ transgenic mice (eg, JNK2, eNOS, and adaptin- β [Table]). These results suggest that PKC ϵ -dependent cardioprotection requires not only an increase in physi-

ological protein-protein interactions involving PKC ϵ but also the development of new interactions that are absent in the naïve, unprotected state. Finally, our finding that molecules such as HSP 27, HSP 70, and MAPKAPK2, which have been implicated in preconditioning, are recruited to the PKC ϵ complexes in transgenic mice (Table, Figure) provides additional evidence supporting the role of these proteins in cardioprotection.

Conclusions

This is the first investigation to use a functional proteomic strategy to systematically delineate PKC ϵ signaling complexes in the heart. This approach has enabled us to demonstrate physical associations of PKC ϵ with numerous structural, signaling, and stress-responsive proteins in the normal heart and to identify new interactions associated with cardioprotection in PKC ϵ transgenic mice. Many of the proteins found to be physically associated with PKC ϵ in this study were not previously known or suspected to interact with this kinase. Therefore, our results suggest novel, heretofore-unrecognized functions for PKC ϵ in the regulation of a multitude of cellular proteins. Based on our findings, we propose that PKC ϵ forms different complexes at different subcellular locations; further studies will be needed to define the composition of individual PKC ϵ complexes in each specific subcellular compartments (eg, nuclei, mitochondria, etc). The recruitment of molecules into a signaling complex is known to be a mechanism to facilitate the interaction of these proteins and the integration of signal transduction. By deciphering the molecular infrastructure that underlies PKC ϵ -dependent signaling in normal and protected myocardium, our observations provide the indispensable framework for future studies aimed at interrogating the functional significance of the observed protein-protein interactions. The information obtained with this proteomic analysis will expedite our understanding of PKC ϵ -dependent cardioprotection and signaling. Given the multiplicity of PKC ϵ functions, this study has broad implications for numerous biological processes in which PKC ϵ has been implicated.

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References

1. Liu GS, Cohen MV, Mochly-Rosen D, Downey JM. Protein kinase C- ϵ is responsible for the protection of preconditioning in rabbit cardiomyocytes. *J Mol Cell Cardiol.* 1999;31:1937–1948.
2. Cross HR, Murphy E, Bolli R, Ping P, Steenbergen C. Overexpression of PKC ϵ protects the ischemic heart, without attenuating ischemic H⁺ production. *Circulation.* 1999;100(Suppl I):I-490–I-491. Abstract 2586.
3. Ping P, Zhang J, Zheng YT, Li RCX, Guo Y, Bao W, Bolli R. Cardiac targeted transgenesis of active PKC ϵ renders the heart resistant to infarction. *Circulation.* 2000;102(Suppl II):II-24–II-25. Abstract 100.
4. Dorn GW II, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN, Mochly-Rosen D. Sustained in vivo cardiac protection by a rationally designed peptide that causes ϵ protein kinase C translocation. *Proc Natl Acad Sci U S A.* 1999;96:12798–12803.
5. Hunter T. Signaling—2000 and beyond. *Cell.* 2000;100:113–127.
6. Pandey A, Mann M. Proteomics to study genes and genomes. *Nature.* 2000;15:405:837–846.