Regulation of Phospholipase C-β3 Activity by Na⁺/H⁺ Exchanger Regulatory Factor 2*

Received for publication, February 21, 2000, and in revised form, March 14, 2000
Published, JBC Papers in Press, March 16, 2000, DOI 10.1074/jbc.M001410200

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Among the phospholipase C that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, four mammalian phospholipase C-β (PLC-β) isotypes (isotypes 1–4) are activated through G protein-coupled receptors (GPCRs). Although the regulation of the PLC-βs by GPCRs and heterotrimeric G proteins has been extensively studied, little is known about the molecular determinants that regulate their activity. The PLC-β isozymes carry a putative PSD-95/Dlg/ZO-1 (PDZ) binding motif (X(S/T/X)(V/L))COOH at their carboxyl terminus, which is implicated in specific interactions with anchor proteins. Using the yeast two-hybrid system, we identified Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2) as a protein that interacted with a C-terminal heptapeptide of PLC-β3. Immunoprecipitation studies revealed that NHERF2 interacts specifically with PLC-β3, but not with other PLC-β isotypes. Furthermore, PLC-β3 interacted with NHERF2 rather than with other PDZ-containing proteins. This interaction required the COOH-terminal NTLQ sequence of PLC-β3 and the second PDZ domain of NHERF2. Interestingly, NHERF2 potentiated the PLC-β activation by carbachol in COS7 and HeLa cells, while mutant NHERF2, lacking the second PDZ domain, had no such effect. Taken together, the data suggest that NHERF2 may act as a modulator underlying the process of PLC-β-mediated signaling.

The binding of GPCRs‡ with their cognate agonists, such as bradykinin, acetylcholine, histamine, and bombesin, sets in motion the activation of phospholipase C-β (PLC-β) isozymes through heterotrimeric G proteins (1). Activated PLC-β catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate and thereby generates two second messengers, diacetyl glycerol and inositol 1,4,5-trisphosphate (2).

Among the heterotrimeric G proteins, the Gαq family activates PLC-β upon stimulation with ligands (3). Likewise, the βγ subunit, which dissociates from the Gαq family, activates PLC-β in a pertussis toxin-sensitive manner (4). Currently, four isotypes of PLC-β have been identified in mammals. Each isotype exhibits a different sensitivity to the Gαq family and βγ subunit. It has been reported that the GTPγS-activated Gαq or Gαq11 subunits stimulated PLC-β isotypes with the ranking order of potency: PLC-β1 ≥ PLC-β3 > PLC-β2. PLC-β4 was also activated by the Gαq subunit (5). The Gβγ subunit activated PLC-β in vitro according to the following hierarchy: PLC-β3 > PLC-β2 > PLC-β1 (6, 7). However, several in vivo studies have shown that the receptors of chemotaxtractants including interleukin-8, C5a, and formyl-Met-Leu-Phe can specifically activate PLC-β2 via the Gβγ subunits from Gαq in a pertussis toxin-sensitive manner (8–10). Gαq and Gβγ subunits may thus independently modulate each PLC-β isotype in cooperation with other intracellular factors.

The distribution of the PLC-β isotypes has been investigated in several tissues and cells. In the brain, PLC-β1 is highly expressed in the cerebral cortex and hippocampus, and PLC-β4 is highly expressed in cerebellum. PLC-β3 is expressed throughout the brain (11–13). The expression patterns of the PLC-β isotypes in different cell lines are also different (14). While PLC-β3 is expressed in most cells, PLC-β2 is dominant in hematopoietic cells (14, 15). All isotypes except PLC-β2 are detected in PC12 cells.² These findings suggest the possibility that each PLC-β isotype plays a distinct role in various GPCR signaling events.

PLC-β isotypes have a long C-terminal regions (−400 residues) that have relatively low homology among them (7). In studies using mutants with deletions in the C-terminal region of PLC-β1, it was determined that the segment (residues 903–1142) was required for binding and stimulation by Gαq (17). Moreover, there are short consensuses sequences known as postasynaptic density-95/discs large/ZO-1 (PDZ)-binding motifs that consist of the amino acids X(S/T)(X/V/L)-COOH at the immediate C terminus of the PLC-β3 isotype (18). PDZ domains exist in a large number of multifunctional proteins that mediate protein-protein interactions at the postasynaptic density in neurons and junctional complexes in epithelia (19, 20). PSD-95/SAP90, which contains three PDZ domains, seems to assemble a receptor and channel complex by interacting with a

* This work was supported in part by Brain Science and Engineering Research Program Grant 98-J04-02-01-A-03, Korea Research Foundation Grant BSRI-98-4443, the National Creative Research Initiative Program of the Ministry of Science and Technology; the Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling Research at Ewha Womans University; POSTECH Research Fund (Korea); and National Institutes of Health Grant DK-44484. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; PLC, phospholipase C; GTPγS, guanosine 5′-3′-O-thiotriphosphate; PDZ, PSD-95/Dlg/ZO-1; PSD-95, postasynaptic density-95; HA, hemagglutinin; NHERF, Na⁺/H⁺ exchanger-regulating factor; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactosopyranoside.

² J.-I. Hwang, K. Heo, K.-J. Shin, E. Kim, C.-H. C. Yun, S. H. Ryu, H.-S. Shin, and P.-G. Suh, unpublished data.
NHERF2 Binds PLC-β3

16633

type of glutamate receptor (N-methyl-D-aspartate receptor), and a shaker type K+ channel and neuronal nitric-oxide synthase (21,22). GRIP has seven PDZ domains and interacts with the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor, another type of glutamate receptor potential through the fourth and fifth PDZ domains (23). The phototransduction pathway in the compound eye of Drosophila is spatially organized by inactivation no afterpotential D, a five-PDZ-containing protein. By interacting with PLC-β, eye-specific protein kinase C and light-activated Ca2+ channel transient receptor potential through its PDZ domains, NAD enables the visual signaling cascade through rhodopsin to be efficiently regulated (24, 25). These reports suggest that the PDZ-containing proteins may involve in various G protein-coupled receptor-mediated signaling pathways. Therefore, it is reasonable to assume that this motif may provide the PLC-β isotypes with specificity in the signal transduction pathway by allowing them to interact with various PDZ-containing proteins.

In this study, we identify some of the proteins interacting with PLC-β using the yeast two-hybrid system. Here, we show that NHERF2 (Na+/H+ exchanger-regulating factor) interacts with the C terminus of PLC-β3 via the second PDZ domain. This interaction is isotype-specific in that the PLC-β3 binds specifically to NHERF2 rather than other PLC isotypes. Furthermore, NHERF2 enhances the activation of PLC-β induced by carbachol treatment. These results suggest that NHERF2 may play a pivotal role in the organization and modulation of the PLC-β-mediated signaling.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—Two-hybrid screening was performed using the L40 yeast strain harboring His3 and β-galactosidase as reporter genes as described previously (26, 27). DNA oligomers encoding the C-terminal heptapeptides of PLC-β isotypes were inserted into pBHA (LexA fusion vector) and used as bait to screen a human fetal liver cdNA library inserted into the activation domain of GAL4 in pGAD10 (CLONTECH, Palo Alto, CA). We screened ~1 × 109 primary transformants for interacting proteins. Clones specifically interacting with the bait were identified by His growth and X-gal activity assay. The DNAs of positive clones were sequenced, and the sequences were compared with sequences in the data bank using the National Center for Biotechnology Information (NCBI) BLAST.

Plasmid Constructs, Site-directed Mutagenesis—In order to express NHERF2 in Escherichia coli, the cDNA of human NHERF2 was inserted into the glutathione S-transferase (GST) fusion vector, pGEX-4T (Amersham Pharmacia Biotech). Various constructs of NHERF2 were provided by Dr. C. H. Yun from Johns Hopkins University (28), and HA-tagged NHERF2 was kindly provided by Dr. Robert Lefkowitz from Duke University (29). GST or His6 fusion proteins were expressed in E. coli BL21 by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 27 °C. Bacterial lysates were prepared by sonication in ice-cold PBS in the presence of protease inhibitors. Proteins were purified from the soluble fraction on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for GST fusion proteins or Ni2+-nitrilotriacetic acid resin (Invitrogen) for His6 fusion proteins. Full-length NHERF2(1–337) and ERM binding domain-deleted NHERF2(1–311) were constructed from the pET30a-NHERF2 by polymerase chain reaction with primers (5′-CGCGCCGGAGCCG-3′ and 5′-CGTCTAGATCGCCGCGGACACT-3′) and 5′-CGGCGGGGACACTTCAG3′ and 5′-CGGCCGCGCCGGACACTTCAG3′ and 5′-CGGCCGCGCCGGACACTTCAG3′. The fragments were then ligated at their SalI and XhoI sites, respectively. The cDNA was then inserted into the expression vector pET30a-NHERF2. PLC-β isotypes and their FLAG-tagged forms in pCDNA3.1 and pCMV2 were constructed from the cdNA of each of the previously isolated PLC-β1 isotype. Each of the individual residues of the PDZ-binding motif (NTQL) of PLC-β3 was mutated to Ala codons and confirmed by DNA sequencing.

Antibodies—For production of NHERF2 antibodies, His6 fusion protein of the entire NHERF2 was purified and used for immunization of mice. After four immunizations, spleenocytes were fused with myeloma cells at a ratio of 10:1 using polyethylene glycol 1500. The hybrids were selected in HAT-supplemented plate as described previously and plated in Eagle’s medium supplemented with 10% fetal bovine serum and selected with hypoxanthine/aminopterin/thymidine. Supernatants of the hybrids were used to screen the pools releasing the antibody for NHERF2. Positive hybridomas were cloned twice by limit dilution. Large quantities of specific monoclonal antibodies were produced by preparation of ascites fluid in Balb/c mice. Purified antibodies were used for immunological studies.

RESULTS

Identification of the PLC-β3-interacting Protein—The C-terminal heptapeptide (QENTQL-COOH) of PLC-β3 was used as

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the bait in the yeast two-hybrid assay screening for proteins interacting with PLC-β3. Two positive clones from a human liver cDNA library were obtained and sequenced. They were found to contain the fragment of NHERF2 extending from the second PDZ domain to the C-terminal region. NHERF2 initially cloned by Yun et al. has two highly conserved PDZ domains, PDZ1 and PDZ2. These domains, which have been described as modules mediating protein-protein interaction in the submembranous region, recognize the C-terminal consensus sequence (X/S/T)(V/L)-COOH of target molecules. The binding specificity of the C-terminal motif of PLC-β3 for various different PDZ domains was examined with a yeast two-hybrid assay (Table I). The PLC-β3 motif interacted specifically with the PDZ2 of NHERF2 but not with the motif in PSD-95 and NHERF1, suggesting that the PLC-β3 binding is specific for NHERF2.

PLC-β3 Specifically Interacted with NHERF2—To confirm the association of PLC-β3 with NHERF2, GST-NHERF2 expressed in E. coli strain BL21 was used in a pull-down assay of COS7 cell lysates. Immunoblot analysis using an anti-PLC-β3 antibody showed that PLC-β3 precipitated with GST-NHERF2 but not with GST alone (Fig. 1A). The extracts of COS7 cells expressing FLAG-PLC-β3 or FLAG-PLC-β3C (C-terminal four amino acids deleted) were incubated with GST-NHERF2. Whole PLC-β3 was detected by the anti-FLAG antibody in the precipitate, whereas the C-terminally deleted form was not. This suggests that PLC-β3 interacted with NHERF2 via the C-terminal PDZ binding motif (Fig. 1B).

NHERF1/EBP50 cloned by Reczek et al. shares high amino acid sequence homology with NHERF2 (32). Both isoforms of NHERF regulate the activity of NHE3 and the cystic fibrosis transmembrane conductance regulator in membranes. We examined whether NHERF1 would also interact with PLC-β3. Extracts of COS7 cells co-expressing FLAG-PLC-β3 together with HA-tagged NHERF1 or Hisα-tagged NHERF2 were allowed to immunoreact with anti-FLAG antibody. Subsequently, the immunocomplexes were analyzed with appropriate antibodies. As shown in Fig. 2, only NHERF2 was co-precipitated with PLC-β3, suggesting that PLC-β3 interacted specifically with NHERF2.

All PLC-β isotypes have the consensus sequence of the PDZ binding motif at their C terminus, which enables the PLC-βs to associate with PDZ domain-containing proteins. We tested the interaction of NHERF2 with each PLC-β isoform by co-expressing NHERF2 with each isoform in COS7 cells (Fig. 3). When NHERF2 was precipitated from cellular extracts using an anti-NHERF2 antibody (antibody 2570), only PLC-β3 was detected in the precipitates (Fig. 3A). In parallel, immunoprecipitation assays using anti-FLAG antibody were performed on extracts of COS7 cells expressing NHERF2 and one of each FLAG-tagged PLC-β isotype. NHERF2 was detected in the immunoprecipitate together with FLAG-PLC-β3, but not with the other isotypes (Fig. 3B). These data suggest that NHERF2 specifically interacts with PLC-β3, although a weak association with PLC-β2 was also detected (Fig. 3, A and B).

The C-terminal Thr and Leu Residues of PLC-β3 Are Essential for the Interaction with NHERF2—It is known that PDZ domains bind to C-terminal four amino acids of target proteins. Ser or Thr at the -2 position and Leu or Val at the terminal position of the PDZ binding motif are essential for the interaction with PDZ domains, while other residues are less effective (33). The binding preferences of NHERF2 were determined by mutating in turn the last four amino acids (NTQL-COOH) of PLC-β3 to Ala. The extracts of COS7 cells co-expressing each FLAG-tagged, mutant PLC-β3 and NHERF2 were reacted with anti-FLAG antibody, and the precipitate was then probed with anti-NHERF2 antibody. Mutation of Thr to Ala at the -2 position or Leu to Ala at the last position resulted in complete loss of NHERF2 binding to PLC-β3. In contrast, mutation of the other residues to Ala had no effect (Fig. 4). The results indicate that these Thr and Leu residues of PLC-β3 participate in the interaction with NHERF2.

The Second PDZ Domain of NHERF2 Is Required for Interaction with PLC-β3—In order to determine which regions of NHERF2 take part in the interaction, we expressed and purified wild type as well as various fragments of NHERF2 as Hisα-tagged recombinant proteins as described previously (28). COS7 cell extracts were incubated with the recombinant

| Table I NHERF2 interacts specifically with the PDZ domain binding motif of PLC-β3 |
| C terminus of PLC-β3 (NTQL) | pGAD10 clones | HIS3 | β-Galactosidase |
|-------------------------------|---------------|------|-----------------|
| NHERF1 PDZ1                  | −             | −    | + +             |
| PSD95 PDZ1                   | −             | −    | +              |
| NHERF2 PDZ2                  | ++            | −    | −              |

FIG. 1. Interaction of NHERF2 with PLC-β3. A, interaction of GST-NHERF2 with PLC-β3. The extract of COS7 cells endogenously expressing PLC-β3 was incubated with GST-NHERF2 immobilized on glutathione beads. The precipitates were detected with anti-PLC-β3 antibody. B, PLC-β3 interacts with NHERF2 through its C-terminal PDZ binding motif. The extracts of COS7 cells expressing FLAG-tagged wild type or the C-terminal deleted form of PLC-β3 were incubated with GST-NHERF2, and the precipitated proteins were detected with anti-FLAG antibody.
NHERF2 constructs immobilized on Ni2+-nitrilotriacetic acid beads. The precipitates were then probed with anti-PLC-β3 antibody. As shown in Fig. 5A, entire NHERF2 and fragments containing the PDZ2 domain were associated with PLC-β3. The data are consistent with the results obtained in the yeast two-hybrid system. Although the C-terminal region of NHERF2 by itself did not effectuate the direct binding to PLC-β3, the PDZ2 domain containing this region associated more tightly with PLC-β3 than PDZ2 alone (Fig. 5A).

To confirm the above results, we undertook further immunoprecipitation experiments. The ERM binding domain-deleted form and the PDZ2-deleted form of NHERF2 were generated as described under “Experimental Procedures.” The cell extracts containing FLAG-PLC-β3 and one of each recombinant NHERF2 were immunoprecipitated with anti-FLAG antibodies. Subsequently, the presence of NHERF2 bound to PLC-β3 was analyzed by immunoblot assay with anti-NHERF2 antibody. As shown in Fig. 5B, both wild type and the ERM binding domain-deleted form of NHERF2 associated with PLC-β3, but the PDZ2-deleted form did not, suggesting that PDZ2 is essential for the interaction.

NHERF2 Increases Phosphoinositides Hydrolysis Induced by Carbachol—COS7 cells transfected with the genes for muscarinic receptor type 1 and various recombinant forms of NHERF2 were labeled with [3H]inositol as described under “Experimental Procedures.” After 12 h, the cells were treated with carbachol, and inositol phosphate release into the cytosol was measured. As shown in Fig. 6A, the accumulation of inositol phosphates upon carbachol treatment increased 3–4-fold in the wild-type NHERF2-transfected cells relative to vector-transfected cells. On the other hand, the PDZ2-deleted NHERF2 had no effect.

In addition, we established HeLa cell lines expressing wild type or deletion mutants of NHERF2. When the cells were fractionated employing a method previously used by Yao et al. (31), both PLC-β3 and wild type NHERF2 were co-localized in the Triton X-100-soluble membrane fraction. The localization of PLC-β3 was not influenced by overexpression of NHERF2, or vice versa. The subcellular localization of the PDZ2-deleted mutant was the same as that of the wild type. The ERM binding domain-deleted form was predominantly localized in the cytosolic fraction, although a small amount of the ERM binding domain-deleted form was detected in the membrane fraction (data not shown). The data suggest that the ERM binding domain may influence the localization of NHERF2 in cells.

The accumulation of inositol phosphates after carbachol treatment in cells expressing wild type or the ERM binding domain-deleted form of NHERF2 was similar to that in the control cells. However, carbachol treatment of cells expressing the PDZ2-deleted form of NHERF2 only barely induced accumulation of inositol phosphates. This suggests that the muscarinic receptor-mediated PLC activity may be regulated by the PDZ2-mediated interaction of NHERF2 with PLC-β3 (Fig. 6B).

**DISCUSSION**

Using the yeast two-hybrid system, we found a molecule that interacted with PLC-β3. Since the C-terminal four amino acids of target proteins are sufficient to serve as the binding site for PDZ domain-containing proteins (33, 34), we used a C-terminal heptapeptide of PLC-β3 as bait and identified NHERF2 as a PLC-β3-interacting protein. NHERF2, also called E3KARP,
FIG. 5. The second PDZ domain of NHERF2 is required for the interaction with PLC-β3. A, PDZ2-containing fragments of NHERF2 interact with PLC-β3. The fragmented forms of His<sub>6</sub>-NHERF2 expressed in BL21 E. coli strain were lysed with 1% Triton X-100 lysis buffer, and the proteins were immobilized on Ni<sup>2+</sup>-nitrilotriacetic acid (NTA) beads. The COS7 cell lysates were then incubated with the immobilized proteins. The bind proteins were resolved by SDS-polyacrylamide gel electrophoresis and then detected with anti-PLC-β3 antibody. PDZ1, the first PDZ domain; PDZ2, the second PDZ domain; PDZ1–2, two PDZ domains; PDZ2-C-ter, the second PDZ domain and C terminus; C42, the second PDZ domain and the C terminus except for the ERM-binding domain. B, PLC-β3 does not interact with the PDZ2-deleted NHERF2. To confirm the in vitro experiment, the extracts of COS7 cells expressing FLAG-PLC-β3 together with each deletion mutant of NHERF2 were immunoreacted with anti-FLAG antibody, and the precipitated proteins were probed with anti-NHERF2 antibody. NH<sub>2</sub>ΔERM, ERM binding domain-deleted NHERF2; NH<sub>2</sub>ΔPDZ2, PDZ2-deleted form; IP, immunoprecipitation.

was initially cloned by two-hybrid screening using the C-terminal tail of the Na<sup>+</sup>/H<sup>+</sup> exchanger type 3 (NHE3) (35). Because this protein was required for the cAMP-dependent inhibition of NHE3, it was thought to be a regulator of NHE3 (28). However, further studies revealed that NHERF2 does not act by itself but that it takes part in the regulation of NHE3 by anchoring other regulators. Recent studies have shown that NHERF can target PKA to NHE3 by associating with ezrin, a member of the ERM family, via the ERM-binding domain in its C-terminal region (36). By analogy, we suggest that NHERF2 may act as a scaffold in PLC-β-mediated signal transduction.

To date, two isotypes of NHERF, NHERF1 (EBP50) and NHERF2 (E3KARP), have been cloned (29, 35). These proteins share 52% amino acid sequence identity. They also have two in tandem PDZ domains and an ERM domain in common. In addition, it has been suggested that they play common roles in linking the cytoskeletal proteins to the plasma membrane. The ERM-binding domain interacts with ERM family proteins such as ezrin, radixin, and moesin, which associate with the cytoskeleton (36, 37). Moreover, the ERM-binding domain interacts with activated ezrin suggests that the molecular interaction may be influenced by intracellular signaling events (38, 39). NHERF2 also associates with NHE3 via the second PDZ domain and other C-terminal region (28). They interact with the four C-terminal residues of cytoplasmic domains of the β<sub>2</sub>-adrenergic receptor and the cystic fibrosis transmembrane conductance regulator through their first PDZ domain (29, 37).

On the other hand, recent reports have shown that NHERF1 is phosphorylated by G protein-coupled receptor kinase 6A under basal conditions, while NHERF2 is not phosphorylated at all (36, 40). Our results show that NHERF2 interacts with PLC-β3, NHERF1 does not. These observation raise the possibility that each member of the NHERF family may be involved in the separate cellular events, although they sometimes have target molecules in common.

With respect to the PLC-β isotypes, immunoprecipitates from extracts of cells co-expressing each PLC-β isotype and NHERF2 showed that NHERF2 specifically interacted with PLC-β3 but only barely with other types. Hall et al. suggested that the C-terminal motif (DTPL-COOH) of PLC-β1 might be a target site of NHERF (33). In the yeast two-hybrid system, NHERF2 also associated with the motif of PLC-β1 (data not shown). However, NHERF2 failed to interact with PLC-β1 in vivo binding assays, indicating that the specificity of an interaction through the PDZ domain may be determined by other regions as well as the binding motif itself. These data also suggest that the PLC-β isotypes act in different signaling pathways, although they share the common ability to hydrolyze phosphatidylinositol 4,5-bisphosphate.

Studies of the molecular interactions of proteins with PDZ domains have shown that the PDZ domains interact with specific carboxyl termini carrying the consensus sequence of X(S/T)X(V/L)/D. The PDZ domain of cortactin-binding protein 1 and PICK1 interact with the C terminus of the somatostatin receptor (QTSI-COOH) and protein kinase C-α (QSAV-COOH), re-
NHERF2 Binds PLC-\(\beta_3\)

In summary, the C-terminal sequence in PLC-\(\beta_3\) and the C-terminal region of NHERF2 form a unit when they interact with PLC-\(\beta_3\). Furthermore, other regions of PLC-\(\beta_3\) and the C-terminal region of NHERF2 may also be involved in the interaction. The isoelectric point of the interaction between PLC-\(\beta_3\) and NHERF2 shows that PDZ domain-containing proteins allow signaling molecules to interact with PLC-\(\beta_3\). This result suggests the possibility that NHERF2 may facilitate the regulation of the PLC-\(\beta_3\) activity by forming complexes with certain signaling molecules. Further investigations are required to determine how NHERF2 organizes the molecules participating in signal transduction and the cross-talk between signaling pathways.

Acknowledgments—We thank Drs. S. Gilmore (Auckland University School of Medicine) and C. H. Heldin (Ludwig Institute for Cancer Research) for critical comments on the manuscript.

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J. Biol. Chem. 2000, 275:16632-16637.
doi: 10.1074/jbc.M001410200 originally published online March 16, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001410200

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