Pleckstrin 2, a Widely Expressed Paralog of Pleckstrin Involved in Actin Rearrangement*

(Received for publication, April 7, 1999, and in revised form, June 8, 1999)

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We have identified a cDNA for pleckstrin 2 that is 39% identical and 65% homologous to the original pleckstrin. Like the original pleckstrin 1, this protein contains a pleckstrin homology (PH) domain at each end of the molecule as well as a DEP (Dishevelled, Egl-10, and pleckstrin) domain in the intervening sequence. A Northern blot probed with the full-length cDNA reveals that this homolog is ubiquitously expressed and is most abundant in the thymus, large bowel, small bowel, stomach, and prostate. Unlike pleckstrin 1, this newly discovered protein does not contain obvious sites of PKC phosphorylation, and in transfected Cos-7 cells, it is a poor substrate for phosphorylation, even after PMA stimulation. Cells expressing pleckstrin 2 undergo a dramatic shape change associated with actin rearrangement, including a loss of central F-actin and a redistribution of actin toward the cell cortex. Overexpression of pleckstrin 2 causes large lamellipodia and peripheral ruffle formation. A variant of pleckstrin 2 lacking both PH domains still had some membrane binding but did not efficiently induce lamellipodia, suggesting that the PH domains of pleckstrin 2 contribute to lamellipodia formation. This work describes a novel, widely expressed, membrane-associated protein and suggests that pleckstrin 2 may help orchestrate cytoskeletal arrangement.

Pleckstrin homology, or PH,1 domains are amino acid motifs that are capable of binding polyphosphoinositides and regulating protein function (1–6). Frequently, the binding of polyphosphoinositides is controlled by a DEP (Dishevelled, Egl-10, and pleckstrin) domain at each end of the molecule as well as a PH domain in the intervening sequence. A Northern blot probed with the full-length cDNA reveals that this homolog is ubiquitously expressed and is most abundant in the thymus, large bowel, small bowel, stomach, and prostate. Unlike pleckstrin 1, this newly discovered protein does not contain obvious sites of PKC phosphorylation, and in transfected Cos-7 cells, it is a poor substrate for phosphorylation, even after PMA stimulation. Cells expressing pleckstrin 2 undergo a dramatic shape change associated with actin rearrangement, including a loss of central F-actin and a redistribution of actin toward the cell cortex. Overexpression of pleckstrin 2 causes large lamellipodia and peripheral ruffle formation. A variant of pleckstrin 2 lacking both PH domains still had some membrane binding but did not efficiently induce lamellipodia, suggesting that the PH domains of pleckstrin 2 contribute to lamellipodia formation. This work describes a novel, widely expressed, membrane-associated protein and suggests that pleckstrin 2 may help orchestrate cytoskeletal arrangement.

1 The abbreviations used are: PH domain, pleckstrin homology domain; DEP, Dishevelled, Egl-10, and pleckstrin; EST, express sequence tag; kb, kilobase (pairs); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; P3K, phosphatidylinositol 3-kinase; HA, hemagglutinin antigen.

2 These studies were supported in part by Grants P50 HL54500 and P01 HL40387 (to C. S. A.) from the National Institutes of Health. 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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Transfection and Immunoprecipitation—100-mm plates containing Cos-7 cells were transfected using the Ca²⁺ phosphate method. 24 h after transfection, the cells were shocked with 10% glycerol and analyzed again 24 h later. Immunoprecipitation was performed by lysing cells in boiling 1% SDS, 10 mM Tris, pH 7.2, and 2 mM EDTA for 15 min, followed by clarification at 13,000 × g for 15 min. The supernatant was diluted in immunoprecipitation buffer (1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 158 mM NaCl, 10 mM Tris, pH 7.2, 5 mM Na-EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.1% aprotinin. The lysates were precleared of nonspecific proteins by three washes with pansorbin, followed by immunoprecipitation with murine HA-11 anti-HA antibody (Babco, Berkeley, CA).

In Vivo Phosphorylation—34 h after transfection, 100-mm plates containing COS-7 cells transfected with pleckstrin-1, pleckstrin-2, or empty vector were each split into triplicate 60-mm plates. 24 h later, two of each set of three plates were incubated for 2 h with phosphate-free medium and 1 mM/ml 32P-labeled free orthophosphate. PMA was...
RESULTS AND DISCUSSION

Pleckstrin 2 Is a Widely Expressed Pleckstrin Paralog—When platelets are activated by agonists such as thrombin, pleckstrin 1 is phosphorylated by protein kinase C, and then regulates polyphosphoinositide second messenger formation and induces actin rearrangement (3, 17, 18). To look for pleckstrin homologs, we used the original human pleckstrin 1 as the query, and searched for pleckstrin-homologous clones using the frame search option of the Embl-Heidelberg Bioccelerator. This search revealed several potential murine EST clones including: AA051380, AI385784, AA798712, AA403397, and AA008011. Further analysis indicated that these clones were overlapping and represented the same transcript. The mRNA contains an open reading frame of 1107 nucleotides and an ATG codon at positions 54–56 that conforms to Kozak's rules for translation initiation (21). The amino acid sequence predicted from the open reading frame is 353 residues in length, terminating with a TGA codon at nucleotide positions 1113–1115. An alternative splice form was also present that was missing amino acids 107–117 (and the comparable region in pleckstrin 2). This similarity between the regulatory region of pleckstrin 1 (amino acids 107–117) and the comparable region in pleckstrin 2 provides additional evidence that pleckstrin 2 is a distinct protein.

A homology search revealed that the translational product was highly related to human pleckstrin 1. As shown in Fig. 1A, the translational product is 39% identical and 65% homologous over the full length of the protein. Like pleckstrin 1, it contains an amino- and a carboxyl-terminal PH domain plus an intervening DEP domain. When aligned with the corresponding regions in the original pleckstrin, the amino-terminal PH domain is 35% identical and 61% homologous, the DEP domain is 33% identical and 66% homologous, and the carboxyl-terminal PH domain is 57% identical and 82% homologous. Because of the strong primary sequence and organizational homology to pleckstrin 1, we have named this clone pleckstrin 2.

Further analysis of the EST data bank demonstrated several partial human pleckstrin 2 clones including AA226122, AA143492, and AA152444. The amino acid sequence predicted from the long open reading frame from human pleckstrin 2 is completely identical to the amino acid sequence of murine pleckstrin 2 and is 39% identical to the original human pleckstrin. Thus, although the original pleckstrin and pleckstrin 2 are highly homologous, they are clearly different proteins.

Despite overall organizational homology, there is little similarity between the regulatory region of pleckstrin 1 (amino acids 107–117) and the comparable region in pleckstrin 2. This region in pleckstrin 1 contains three sites of phosphorylation, Ser113, Thr114, and Ser117, preceded by a pseudo-substrate site. We tested the ability of pleckstrin 2 to be phosphorylated by protein kinase C, and then regulated polyphosphoinositide second messenger formation and induced actin rearrangement (3, 17, 18). To look for pleckstrin homologs, we used the original human pleckstrin 1 as the query, and searched for pleckstrin-homologous clones using the frame search option of the Embl-Heidelberg Bioccelerator. This search revealed several potential murine EST clones including: AA051380, AI385784, AA798712, AA403397, and AA008011. Further analysis indicated that these clones were overlapping and represented the same transcript. The mRNA contains an open reading frame of 1107 nucleotides and an ATG codon at positions 54–56 that conforms to Kozak's rules for translation initiation (21). The amino acid sequence predicted from the open reading frame is 353 residues in length, terminating with a TGA codon at nucleotide positions 1113–1115. An alternative splice form was also present that was missing amino acids 107–117 (and the comparable region in pleckstrin 2). This similarity between the regulatory region of pleckstrin 1 (amino acids 107–117) and the comparable region in pleckstrin 2 provides additional evidence that pleckstrin 2 is a distinct protein.

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Pleckstrin 2 Is Not an Efficient Substrate of PKC—Pleckstrin 1 is a substrate of multiple isoforms for PKC, and its phosphorylation has been used as an early marker of platelet activation. We have mapped three sites of phosphorylation of pleckstrin 1 (Ser113, Thr114, and Ser117) that are located near its amino-terminal PH domain (24). Although pleckstrin 2 does have a single serine at a position homologous to Ser117 in the original pleckstrin, pleckstrin 2 lacks the conserved PKC pseudo-substrate site. We tested the ability of pleckstrin 2 to be phosphorylated in vivo. Cos-7 cells were transfected with epo-tope-tagged HA-pleckstrin 1 or HA-pleckstrin 2, labeled with 32P, and stimulated with PMA. The HA epitope-tagged proteins were then immunoprecipitated and analyzed for incorporation of radioactive P32. As shown in the left panel of Fig. 2, considerable amounts of HA-pleckstrin 1 and HA-pleckstrin
We previously reported that pleckstrin 1 associates with the cell membrane, Cos-7 cells were transfected with HA epitope-tagged pleckstrin 1 or pleckstrin 2. The left panel shows an anti-HA immunoblot demonstrating roughly equivalent expression and immunoprecipitation of both pleckstrins. The right panel shows the relative phosphorylation of the pleckstrins when the cells were labeled with $^{32}$P-PO$_4$ with and without stimulation with 50 nM PMA. The expressed pleckstrin was immunoprecipitated, fractionated by SDS-PAGE, and autoradiographed.

2 were expressed and immunoprecipitated. However, as seen on the right panel of Fig. 2, whereas pleckstrin 1 incorporated substantial amounts of $^{32}$P, pleckstrin 2, even in the presence of PMA, incorporated little label. In several experiments, a faint phosphoprotein could sometimes be detected in the pleckstrin 2 immunoprecipitates, suggesting that phosphorylation of pleckstrin 2 might occur but is insufficient under the conditions of our experiment. However, we cannot exclude the possibility that pleckstrin 2 is phosphorylated by a kinase other than PKC, one that is not active in our transfected cells. There was no difference in $^{32}$P incorporation when the shorter splice variant of pleckstrin 2 was used instead of the full-length pleckstrin 2 isoform (data not shown).

Also as shown in Fig. 2, the mobility of pleckstrin 2 during PAGE was faster than predicted, and it was faster than the mobility of comparably sized pleckstrin 1. Because the DNA sequencing of pleckstrin 2 was confirmed on several occasions, it is probable that the difference in apparent size reflects an alternative folding or post-translational processing of the protein. Thus, although pleckstrin 2 is highly homologous to pleckstrin 1, there are fundamental differences in the regulation of these proteins.

Pleckstrin 2 Is Membrane-associated and Contributes to Lamellipodia Formation—We previously reported that pleckstrin 1 associates with the plasma membrane in a phosphorylation-dependent fashion (3). To determine whether pleckstrin 2 also associates with the cell membrane, Cos-7 cells were transfected with either wild type pleckstrin 1, wild type, full-length pleckstrin 2, the shorter splice variant of pleckstrin 2, or a mutant form of pleckstrin 2 lacking both PH domains. As shown in Fig. 3, full-length pleckstrin 1 and pleckstrin 2 were membrane-associated. However, there was a difference between the two proteins. Cells overexpressing pleckstrin 1 were flat with some villous projections, and pleckstrin 1 was uniformly distributed over the entire surface membrane including lamellipodia. In contrast, pleckstrin 2-expressing cells had more microvilli and large lamellipodia with ruffle formation. Both types of pleckstrin were predominantly membrane-bound. In contrast, cells expressing the shorter form of pleckstrin 2 were rounder but still had large lamellipodia. The majority of the expressed shorter isoforms of pleckstrin 2 appeared on the membranes of both the cell body and the nucleus but not on the membrane of the lamellipodia. Thus, pleckstrin 1 and both forms of pleckstrin 2 all contribute to lamellipodia formation, but in contrast to the original pleckstrin, pleckstrin 2 induces more microvilli and ruffles.
never been a known non-hematopoietic paralog. In that context, the present studies demonstrate that: 1) like the original pleckstrin, pleckstrin 2 contains a PH-DEP-PH organization; 2) its transcript is most abundant in the thymus, stomach, large and small bowels, and prostate; 3) in contrast to the original pleckstrin, it is not efficiently phosphorylated by PKC; and 4) it induces lamellipodia and ruffle formation. These observations raise a number of issues, including the mechanism by which pleckstrin 2 induces actin redistribution and the mechanism by which pleckstrin 2 is regulated.

The first issue is the mechanism of pleckstrin 2-mediated actin changes. The original pleckstrin also induces cytoskeletal reorganization, although it tends to contribute to less lamellipodia. In the case of the original pleckstrin, many of its effects can be inhibited with a dominant-negative Rac and are independent of PI3K. Whether pleckstrin 2-mediated cytoskeletal changes involve Rac is currently unknown as is the role of other polyphosphoinositide-binding proteins such as gelsolin or villin. The observation that the pleckstrin 2 truncated variants of both PH domains may still bind to the cell membrane was an unexpected finding. Whether the association of this mutant with the cell membrane is mediated by its DEP domain is an area for further study, as are the circumstances that allow different regions of pleckstrin 2 to direct localization to different structures.

The second issue is the mechanism by which pleckstrin 2 is regulated and the related issue of the identity of its potential binding partners. Pleckstrin 1 is phosphorylated by multiple isoforms of PKC, and its ability to induce actin reorganization is regulated by that phosphorylation. Pleckstrin 2 is not efficiently phosphorylated by PKC, and thus it requires an alternative means of regulation. During the preparation of this work, we became aware of the results by E. Skolnik and co-workers (25) who used a genetic screen for proteins that bind D3-containing phosphoinositides. It therefore seems reasonable to speculate that pleckstrin 2 is regulated by PI3,4P2 or PIP3, and may in turn be an effector for PI3K. We are currently investigating whether the intracellular distribution of pleckstrin 2 is affected by PI3K-mediated signaling and whether pleckstrin 2 moderates any PIP2-dependent signaling events such as the activation of Akt, PKC, or mitogen-activated protein kinase.

Acknowledgments—The authors thank Dr. Toby Gibson (EMBL Heidelberg) for the initial alignments on the EMBL-Heidelberg Bioseparator and Drs. Joel S. Bennett and Lawrence F. Brass for their helpful comments on this manuscript.

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doi: 10.1074/jbc.274.31.21515

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